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Detroit, Michigan
August 21-24, 1961

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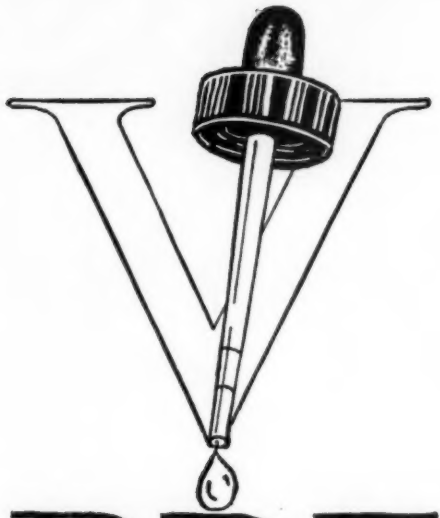

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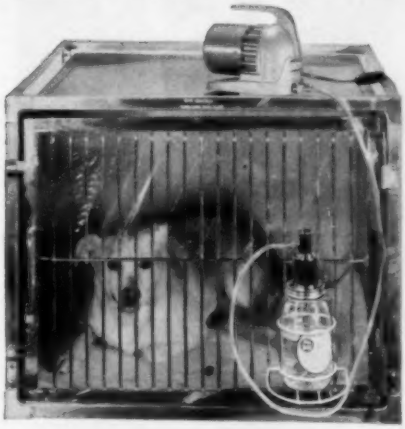

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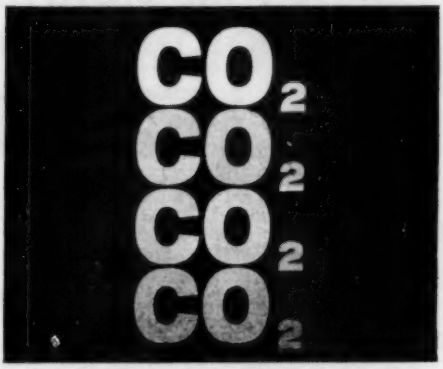

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VOL. XXII MARCH, 1961 No. 87

Placental Pathology of Sheep with Vibriosis

RUE JENSEN, D.V.M., Ph.D.; V. A. MILLER, D.V.M., M.S.;
J. A. MOLELLO, D.V.M., M.S.

Fort Collins, Colorado

VIBRIOSIS, an acute infectious disease of sheep, is characterized by abortion during late gestation and is caused by *Vibrio fetus*. Abortion usually occurs 1 to 3 days following death of the fetus. Intra-uterine decomposition of the placenta, immediately preceding and following death of the fetus, is advanced at the time of abortion. Ovine fetal placentas are indeciduate and separate from the endometrium at the time of expulsion. These two factors preclude the precise determination of gross and histopathologic changes by study of placentas obtained from ewes at the time of abortion.

In this study, 2 experiments were conducted to determine: (1) placental lesions in sheep inoculated intravenously with *V. fetus* and (2) in sheep infected naturally with vibriosis.

REVIEW OF LITERATURE

Normally, according to Arey [1] and Zietzchmann and Krolling,[9] sheep develop a definitive cotyledonary syndesmochorial placenta. Caruncles (groups of endometrial septums) were found to be in apposition to cotyledons (clusters of chorionic villi). Caruncular septums interdigitated with corresponding cotyledonary villi to form an anatomic and physiologic unit for the transfer of some solutes between fetal and maternal blood. The functional combination of caruncle and cotyledon constituted the placentome.

Assheton [2] and Wimsatt [6] studied the gross anatomy and histologic changes of the sheep placenta throughout gestation by killing pregnant sheep at intervals. By the eighteenth day of gestation, the fetal placenta occupied the entire length of the uterus. At this time, destruction of uterine epithelium began at the placentomes and extended subsequently over the entire endometrium. The epithelium regenerated over the interplacentomal tissue by approximately the 70th day and at the caruncles following parturition. At the placentomes, the endometrium, divested of maternal epithelium, formed multiple, thin-walled septums which projected from the endometrial surface and carried maternal blood vessels. Crypts formed between bases of the septums.

Chorionic villi retained epithelium over the entire surface throughout gestation. Primary villi subdivided to form successively smaller branches of the first, second, and third orders. The villi contained a rich fetal vascular system. Lacunas, spaces between the bases of primary villi, corresponded in the chorion to crypts in the caruncle. Endometrial septums interdigitated with the chorionic villi in close apposition. Extremities of the septums, located between the bases of primary villi, underwent hyaline degeneration. Rupture of blood vessels in these areas, beginning at approximately the 70th day of gestation, caused extravasation of maternal blood into the lacunas, forming hematomas which persisted for the remainder of gestation. Binucleated, giant, epithelial cells transferred from the trophoblast to septums, especially in the crypts, to form syncytial epithelial giant cells.

McFadyean and Stockman [5] studied vibriosis of sheep and determined that pathologic changes were limited to the uterus and its contents. In early stages of the disease, no gross alterations were discernible. In advanced stages, the uterine wall was edematous. Exudate was observed between the chorion and endometrium and was often mixed with blood. Cotyledons, normally firm, were soft,

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From the College of Veterinary Medicine and Agricultural Experiment Station, Colorado State University, Fort Collins.

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congested, pulpy, sometimes yellow, and sometimes partially separated from the caruncles.

Biswal *et al.*[3] investigated placental pathologic changes in a flock of 114 sheep affected with vibriosis. Fetal membranes were not retained in the uterus following abortion. Panhysterectomy was performed in 2 infected ewes and 1 normal ewe in order to observe placentas prior to autolysis. In the infected ewes, the placentas were soft. The surface of the endometrium contained exudate and clotted blood. The uterine wall was edematous. Histologically, the changes were not uniform. The endometrium was edematous and infiltrated with leukocytes, especially in the intercaruncular tissue. In the placentomes, the septums were congested and thickened. Chorionic epithelium was necrotic, and extravasation of erythrocytes occurred between septums and villi.

Gilbert[4] studied the placentas of ewes which had aborted due to vibriosis. The placentas contained dark red, intercotyledonary tissue and heavily congested blood vessels. The surface of the membranes contained some gray deposits. Gilbert observed that pathologic changes due to vibriosis were not distinctive.

Smith[6] studied changes in the placentas of 14 cows affected with spontaneous vibriosis. Membranes were retained, and extensive autolysis precluded accurate evaluations. The chorion was edematous. Chorionic epithelium was destroyed and leukocytes infiltrated the tissue. Later, Smith[7] studied 7 additional cows affected with spontaneous vibriosis. Membranes were retained in each of the 7 animals. In the placenta, he observed opaque yellow plaques, a yellow excrescence on half of the chorion, and yellow cotyledons. In 2 pregnant cows affected with vibriosis produced by intravenous inoculation of *V. fetus*, Smith observed placental edema, necrosis of cotyledons, the presence of exudate, and foci of opacity.

TABLE 1—Pregnant Ewes Inoculated Intravenously with Vibrio fetus and Killed 4 to 8 Days Later

Lot No.	No. of ewes	*V. fetus* organisms per ewe	No. ewes remaining normal	Stage of disease at necropsy		
				Early	Advanced	Abortion
1	14	2.3×10^9	2	6	5	1
2	5	none	5	0	0	0

MATERIALS AND METHODS

The changes in the placentas were determined in animals with experimental vibriosis (experiment 1) and compared with placental changes in spontaneous vibriosis (experiment 2).

Experiment 1.—In experiment 1, pregnant sheep were inoculated intravenously with pathogenic *V. fetus* and were subsequently killed to compare diseased placentas with normal placentas.

Sheep.—Nineteen fine-wooled, crossbred, pregnant ewes, approximately 6 years of age, were obtained from a single source in northwestern Colorado. The sheep had been raised and maintained on a ranch where there was no history of vibriosis. The animals were assigned to 2 lots by stratified randomization according to body weight. Ewes of lot 1 were inoculated intravenously, at the beginning of the fifth month of pregnancy, with pathogenic *V. fetus;* animals of lot 2 were not inoculated (table 1). Animals of both lots were provided with the same type of ration and environment and were maintained in isolation subsequent to inoculation of animals of lot 1.

TABLE 2—Ovine Fetal Placentas from Normal Parturition and from Spontaneous Abortions Due to Vibriosis

Source of placentas	No. of placentas	Condition	
		No change	Advanced disease
Normal parturition	5	.....	.....
Spontaneous vibriosis	36	5(14%)	31(86%)

Inoculum.—*Vibrio fetus,* recently isolated from an aborted lamb, was cultivated on blood agar and suspended in buffered physiologic saline solution. Five milliliters of the suspension, which contained a total of approximately 2.3×10^9 viable organisms per inoculation, was injected intravenously into each ewe of lot 1. Pathogenicity of this strain of *V. fetus* had been demonstrated previously by oral administration to pregnant ewes.

Necropsy.—Animals of lots 1 and 2 were killed by transecting the medulla oblongata 4 to 8 days subsequent to inoculation of animals in lot 1. At necropsy, the uterine horns were separated and opened by a longitudinal incision along the lesser curvature. After removal of a fetus, the intact placenta was examined carefully for abnormalities. From each ewe, at least 20 cotyledons were separated manually and aseptically from their respective caruncles. Smears were made from the cotyledons. At least 20 placentomes were excised from each uterus and were fixed in Zenker's solution, 4 per cent formaldehyde in aqueous saline solution, and in Bouin's fluid. Paraffin sections were made from each fixed placentome and were stained with hematoxylin and eosin and with Giemsa's solution. Smears of cotyledons were stained with Giemsa's solution. Photographs were made of typical gross and microscopic lesions.

Bacterial cultures were prepared from each of several cotyledons and from the fetuses of each ewe.

Classification of Infected Placentas.—On the basis of gross and microscopic lesions, placentas from all sheep were classified into 1 of 3 categories: (1) normal, (2) early stages of vibriosis, and (3) advanced stage of vibriosis (table 1).

Experiment 2.—In experiment 2, the changes in fetal membranes from ewes which aborted due to

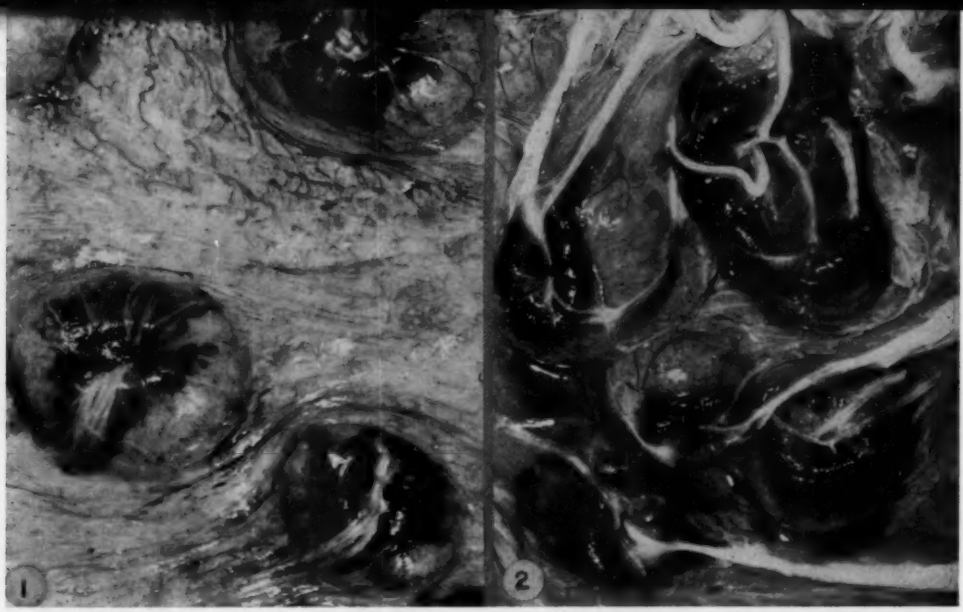

Fig. 1—Normal placentomes, showing hematomas, at the fifth month of gestation. x 1.0.

Fig. 2—Normal placentomes at the beginning of the fifth month of gestation. Hematomas are large. x 1.0.

vibriosis were compared with changes in the placentas of animals of experiment 1 (table 2). Fetal membranes were collected from 5 ewes having normal parturitions and from 36 ewes which aborted due to vibriosis, as determined by isolation of *V. fetus* from aborted fetuses or from diseased membranes. The normal fetal membranes were from ewes in a flock owned by Colorado State University. The 36 diseased fetal membranes were from 6 different commercial flocks which submitted aborted fetuses and membranes to the diagnostic laboratory. All membranes, both normal and diseased, were fixed, sectioned, and stained as described for experiment 1. On the basis of histopathologic changes, membranes were classified as showing advanced disease or no change.

In flocks with a high incidence of abortion and with 1 to 5 per cent mortality of affected ewes, some with fatal cases were necropsied to determine uterine lesions and the cause of death.

Results

In both experiments, diseased placentas had similar changes and, in advanced stages, were readily differentiated from normal placentas.

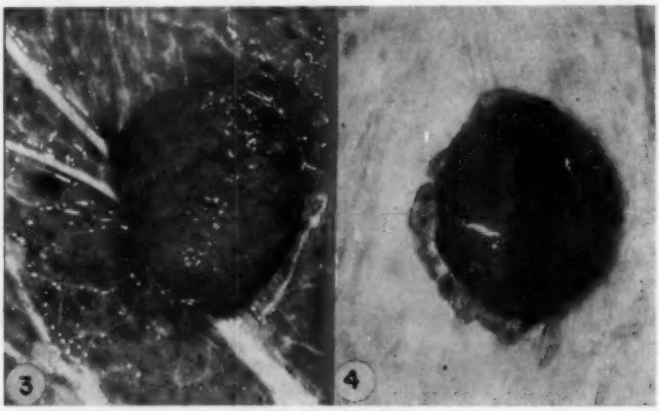

Fig. 3—Normal cotyledon from a sheep killed during the fifth month of gestation. The cotyledon was separated manually from caruncle. x 1.1.

Fig 4—Normal cotyledon from a sheep, following normal parturition. x 1.0.

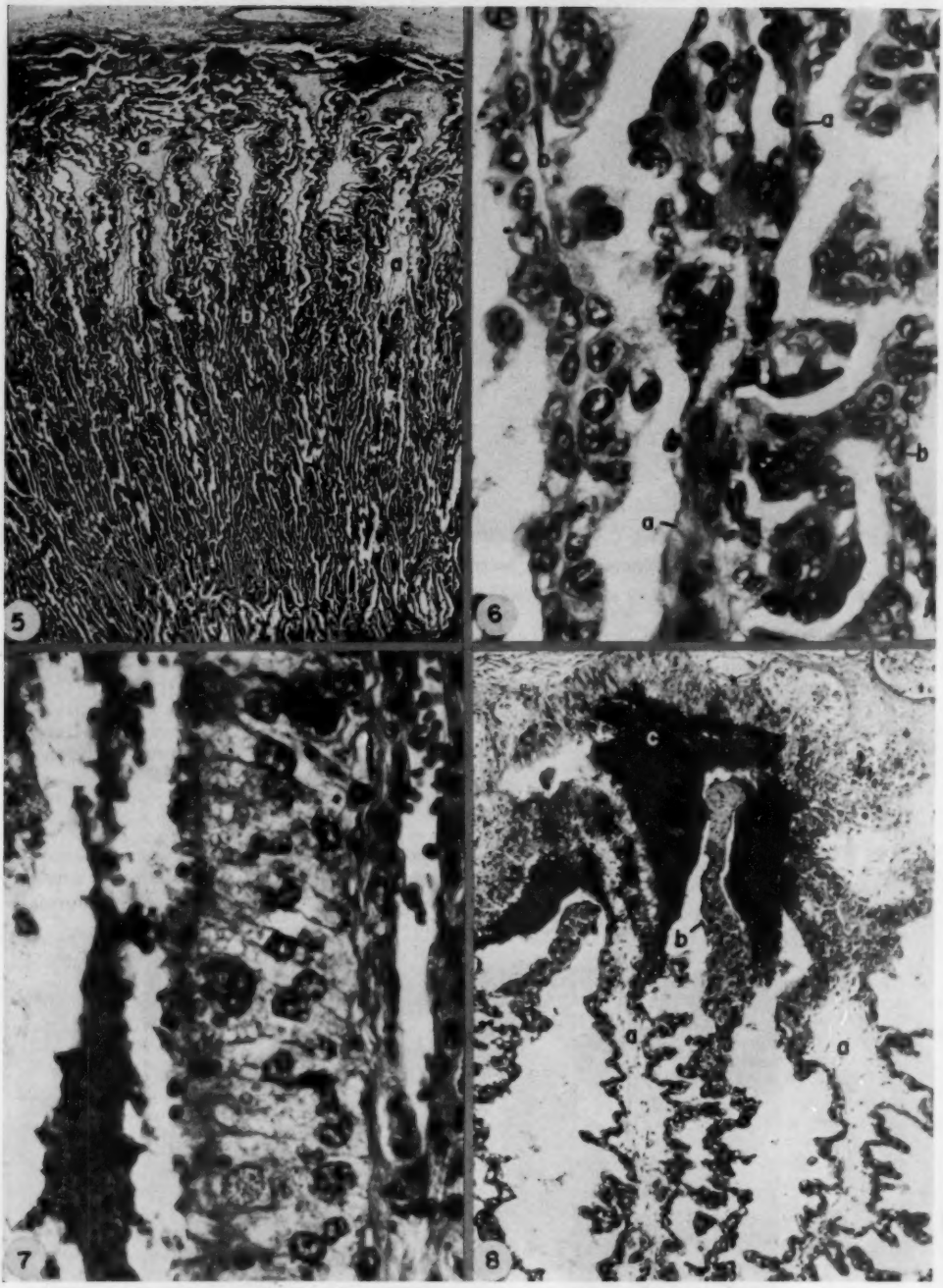

Fig. 5—Normal placentome during the fifth month of gestation. (a) Primary chorionic villi from chorion above; (b) septums from endometrium; and (c) hematoma. H & E stain; x 9.

Fig. 6—Normal placentome during the fifth month of gestation. (a) Septum and (b) villi. H & E stain; x 504.

Fig. 7—Columnar epithelium from an arcade of normal chorion at the fifth month of gestation. At left is blood of a hematoma. Some erythrocytes have been phagocytized. H & E stain; x 520.

Fig. 8—Normal placentome during the fifth month of gestation. (a) Primary villi; (b) septum with dilated and hyalinized extremity; and (c) hematoma. H & E stain; x 75.

EXPERIMENT 1

Classification of Placentas.—At necropsy, of the 14 inoculated ewes, 2 were found to be normal, 6 were in early stages of vibriosis, and 6 were in advanced stages of vibriosis (table 2); of the latter, 1 animal had aborted. The 5 uninoculated ewes were normal at the time of necropsy.

Normal Placentas.—The placentas of the 5 uninoculated ewes were similar. Each uterus contained a single normal fetus. The amnionic surface of each placentome was dark because of the hematomas (fig. 1, 2). Placentomes were firm; manual separation of the cotyledons from the caruncles required considerable force. Cotyledons were pink; normal ones are shown (fig. 3, 4).

Microscopically, the placentas were normal and had the characteristic features of definitive sheep placentas. Intercaruncular endometrium was covered with intact epithelium. Within the placentome, the endometrium formed multiple, thin-walled, branching septums. Epithelium was absent except for the syncytial multinucleated cells. Extremities of septums were hyalinized and, in most cases, surrounded by extravasated maternal blood. Blood vessels were normal. The myometrium was moderately edematous. The multiple-branched chorionic villi were covered with normal trophoblastic epithelium and were interdigitated with endometrial septums. Normal and hemolyzed blood filled much of the space between the chorionic epithelium and septums in the area between bases of primary chorionic villi. Columnar epithelium of the arcades had phagocytized entire

Fig. 9—Normal placentome during the fifth month of gestation. Dotted line encloses the "hilar zone" where primary changes from vibriosis occur. H & E stain; x 5.

erythrocytes and fragments of erythrocytes. The lesions are shown (fig. 5–9).

For convenience, the term "hilar zone" is proposed for the portion of the placentome nearest to the hilus of the cotyledon. On vertical section of the placentome, this zone is the third of the placentome nearest the hilus of the cotyledon (fig. 9) and is the site of important initial changes in vibriosis.

Vibrio fetus was not isolated from the placentas of any noninoculated sheep.

Vibriosis-Affected Placentas of Ewes of Lot 1.—At the time of necropsy, 6 placentas were in early stages of vibriosis and 6 were in advanced stages. Grossly, the former appeared normal. Each uterus contained a single fetus.

Of the 6 animals in advanced stages of vibriosis, 5 contained a single fetus each and 1 contained twin fetuses. Edema, although uneven, was extensive and was readily discerned in the chorion from the amnionic surface and from the cut surface. Sanguineous transudate caused thickening of the intercotyledonary chorion as well as the chorion at the hilus of the cotyledons. Severe edema obscured details of some placentomes. In some animals, the myo-

TABLE 3—Histopathologic Changes in Ewes Inoculated with Vibrio fetus

Change	Stage	
	Early	Advanced
Septal changes		
arteritis	2/6	5/5*
necrosis of extremities	6/6	5/5
leukocytosis	6/6	5/5
Lacunal changes		
V. fetus in hematomas	4/6	6/6
leukocytes in hematomas	6/6	6/6
Chorionic changes		
V. fetus in epithelium	0/6	6/6
V. fetus in capillaries	0/6	6/6
necrosis of epithelium	0/6	6/6
leukocytosis of villi	3/6	6/6
edema		6/6
Edema of myometrium	6/6	6/6

* Caruncles from sheep which aborted were not examined.

Denominator = No. of ewes; numerator = ewes in which there were specific lesions.

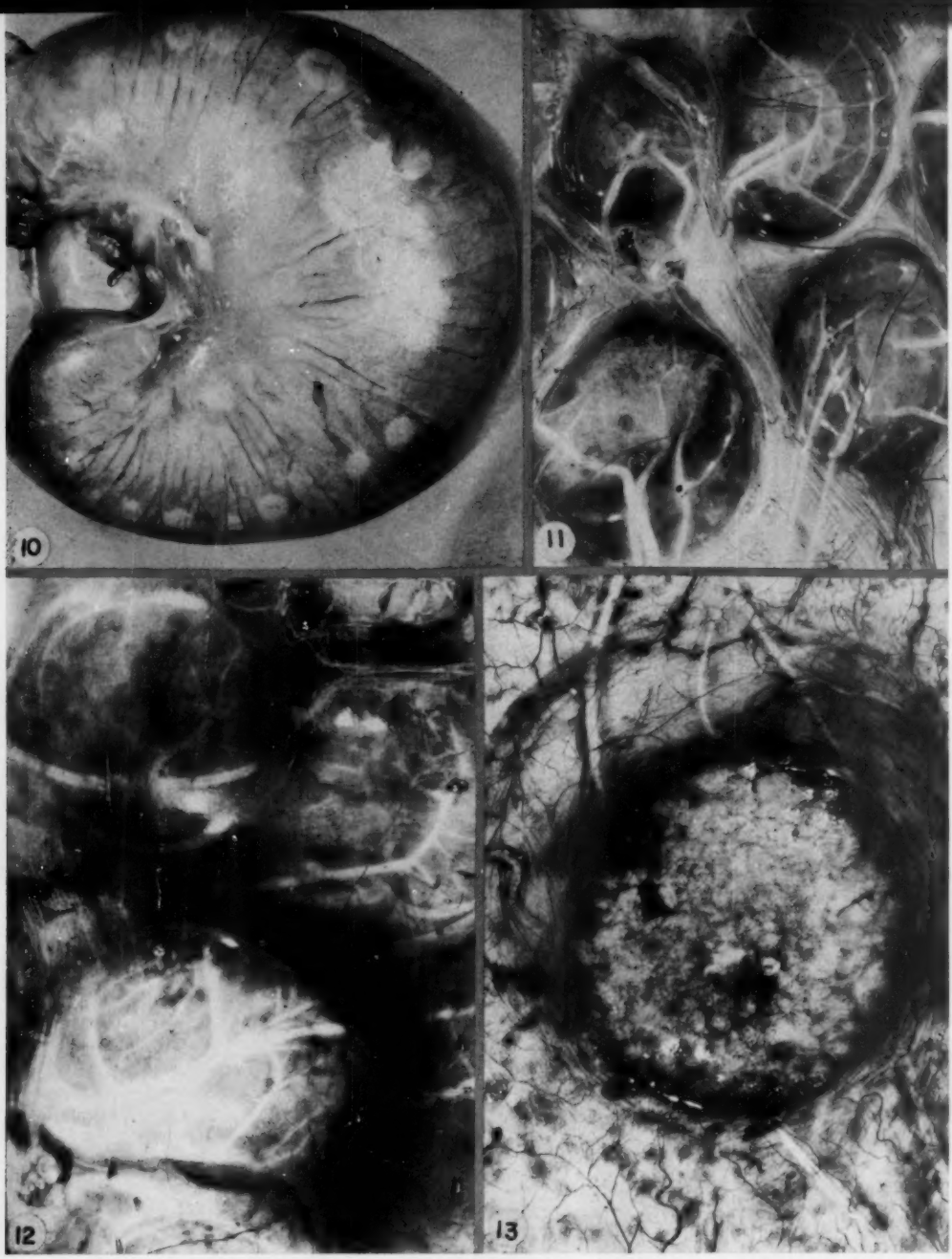

Fig. 10—Edema at lesser curvature of uterus in fifth month of gestation from a sheep affected with vibriosis. x 0.3.

Fig. 11—Edema in chorion over and between placentomes from a sheep inoculated with Vibrio fetus during fifth month of gestation. x 10.

Fig. 12—Severe edema in chorion between placentomes from a sheep inoculated with Vibrio fetus during fifth month of gestation. x 10.

Fig. 13—Cotyledon, separated manually from caruncle, from an experimental sheep with advanced vibriosis during fifth month of gestation. Cotyledon is pale and pericotyledonary chorion is edematous. x 1.0.

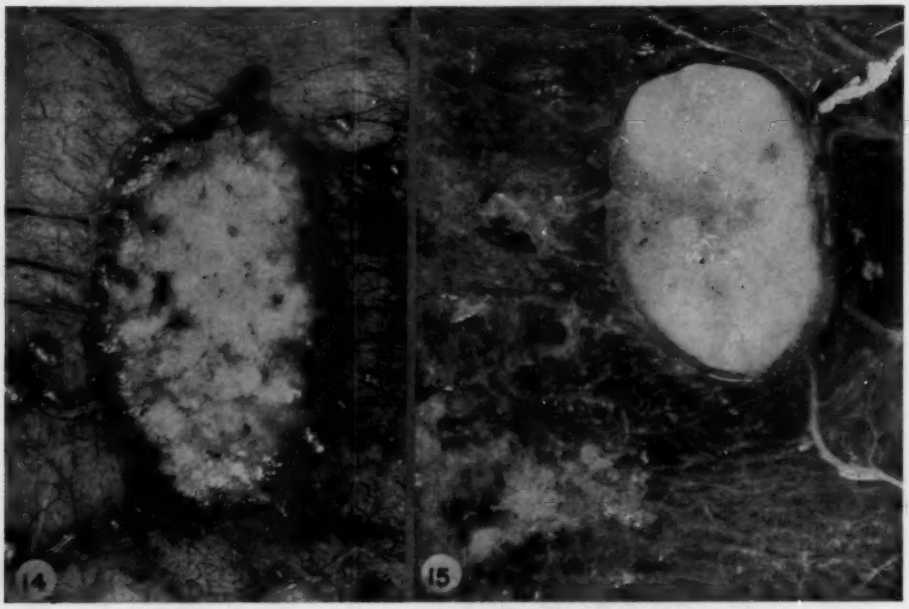

Fig. 14—Cotyledon, separated manually from caruncle, from an experimental sheep with advanced vibriosis during fifth month of gestation. Infected cotyledon is pale. x 1.0.
Fig. 15—Chorion, separated manually from caruncle, from an experimental sheep with advanced vibriosis during fifth month of gestation. Cotyledon is pale. The surface of intercotyledonary tissue contains exudates. x 1.0.

metrium, especially at the lesser curvature of the horn, was edematous from the serosal surface and from the cut surface.

The placentomes were soft and cotyledons were separated easily from caruncles by digital manipulation. Some cotyledons separated from respective caruncles as a result of careful manipulation, without purposeful traction on the membranes, for removal of fetuses. The separated cotyledons were soft and often pale yellow. Flakes of semisolid sanguineous debris were discernible in the interplacentomal areas between endometrium and chorion. Gross changes are shown (fig. 10–16).

The uterus with twin fetuses had uneven development of vibriosis in the 2 horns. The placenta of 1 horn appeared to be normal grossly but, histopathologically, it was shown to be in early stages of vibriosis. The placenta of the other horn had gross changes characteristic of advanced vibriosis.

Histopathologic changes in placentomes of placentas in early vibriosis were limited to the hilar zone. In multiple foci, the septums, especially towards extremities,

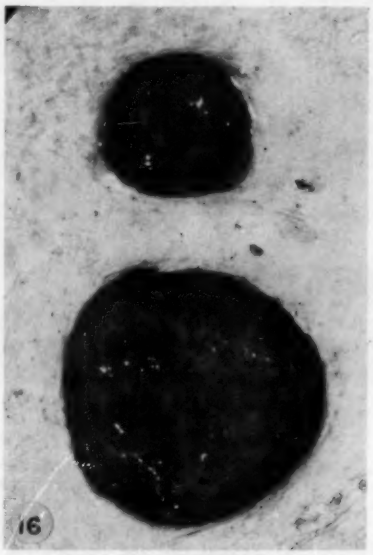

Fig. 16—Cotyledons, separated manually from caruncles, from an experimental sheep with early vibriosis in fifth month of gestation. x 1.0.

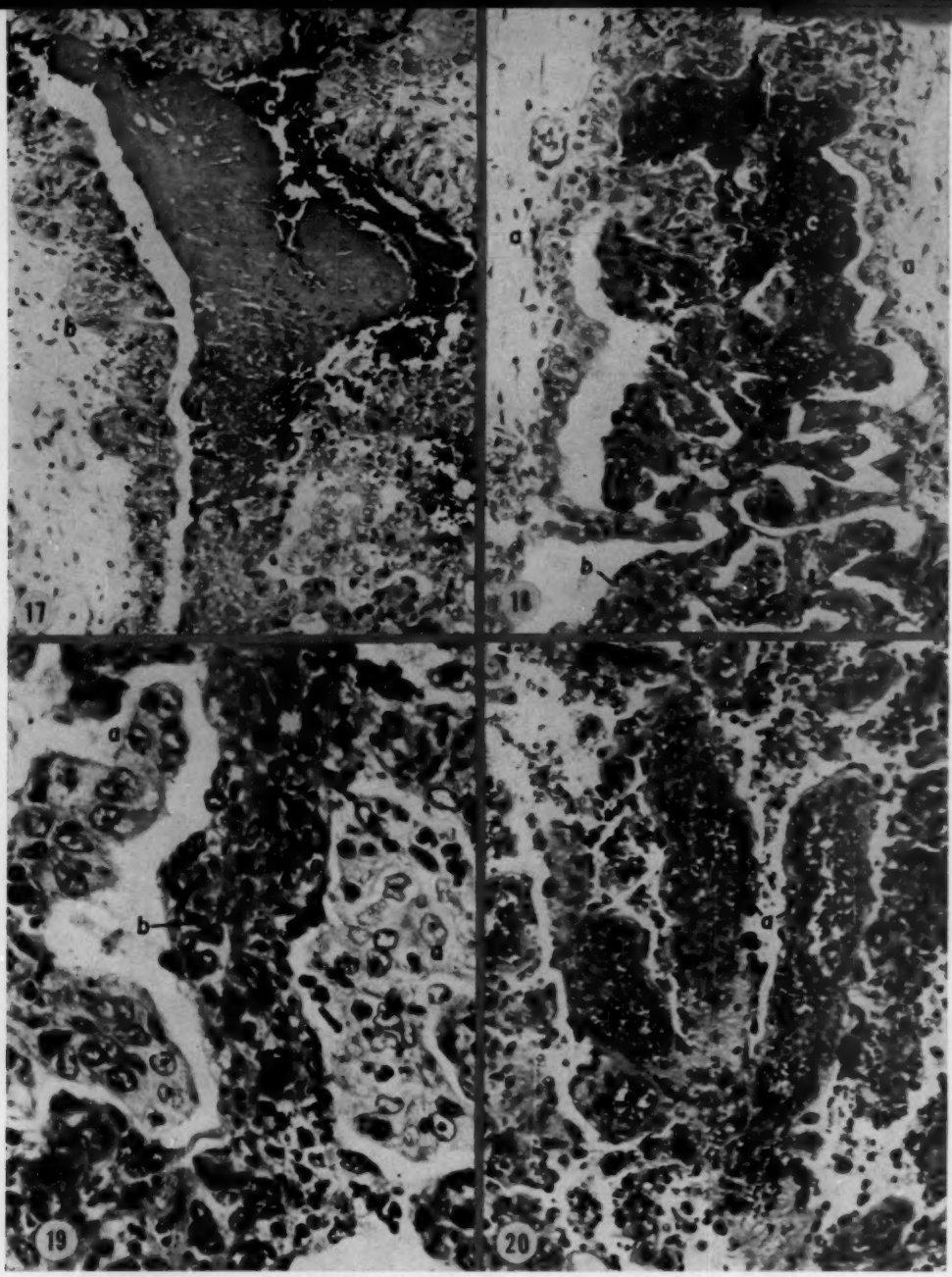

Fig. 17—Placentome in early stage of vibriosis from sheep in fifth month of gestation. (a) Necrosis and leukocytes in a focus of infection in extremity of septum; (b) chorion; and (c) hematoma. H & E stain; x 126.

Fig. 18—Placentome in early vibriosis from experimental sheep in fifth month of gestation. (a) Normal chorion; (b) normal portion of septums; and (c) necrosis, leukocytosis, and thrombosis of infected septal extremity. H & E stain; x 156.

Fig. 19—Placentome in early vibriosis from experimental sheep in fifth month of gestation. (a) Normal villi and (b) necrosis and leukocytosis of septum. H & E stain; x 504.

Fig. 20—Septums from placentome in early vibriosis from experimental sheep in fifth month of gestation. (a) Arteriolitis, with necrosis and thrombosis. H & E stain; x 43.

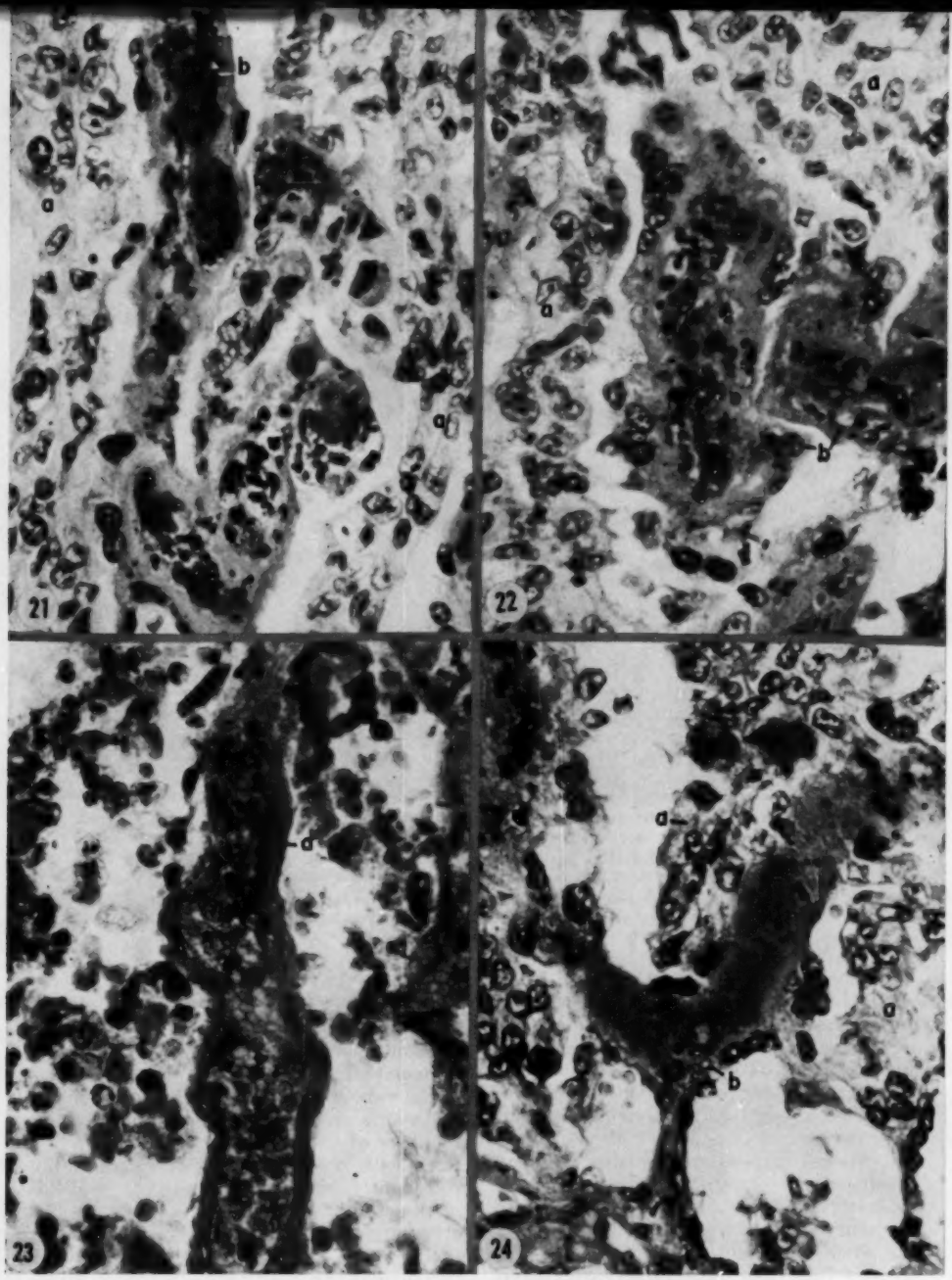

Fig. 21—Placentome in early vibriosis from experimental sheep in fifth month of gestation. (a) Normal villi and (b) septum with necrosis and thrombosis. H & E stain; x 504.

Fig. 22—Placentome in early vibriosis from experimental sheep in fifth month of gestation. (a) Normal villi and (b) septal capillaries with necrosis and thrombosis. H & E stain; x 504.

Fig. 23—Septum from placentome in early vibriosis from sheep in fifth month of gestation. (a) Necrosis in wall of capillary. H & E stain; x 550.

Fig. 24—Placentome in early vibriosis from experimental sheep in fifth month of gestation. (a) Normal villi and (b) thrombosis of septum. H & E stain; x 504.

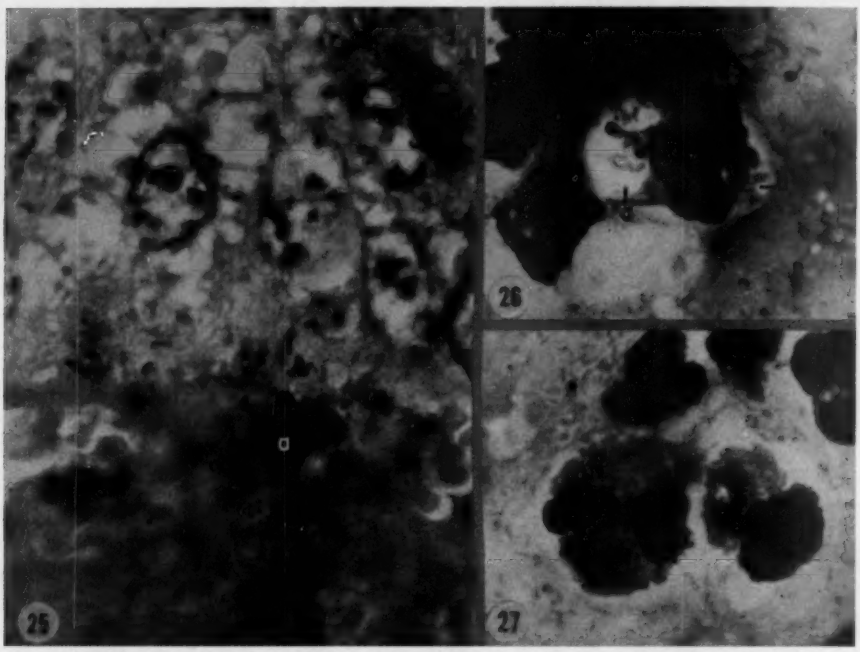

Fig. 25—Vibrio fetus penetrating chorionic epithelium of placentome in early vibriosis from experimental sheep in fifth month of gestation. (a) Blood of hematoma. Giemsa stain; x 1,260.

Fig. 26—Smear from cotyledon in early vibriosis from experimental sheep. (a) Vibrio fetus phagocytized by mononuclear leukocytes. Giemsa satin; x 1,536.

Fig. 27—Smear from cotyledon from experimental sheep in early stages of vibriosis. Vibrio fetus has been phagocytized by mononuclear leukocytes. Giemsa stain; x 1,536.

were necrotic and infiltrated with leukocytes. In these areas, leukocytes were also in the blood of hematomas. *Vibrio fetus* organisms were discernible in extravasated blood of affected areas. Incipient infiltration with leukocytes of adjacent chorion was often present. Endometrium immediately peripheral to points of attachment of placentomes to myometrium was inflamed. Uterine glands contained exudate and the endometrium was infiltrated with polymorphonuclear leukocytes, lymphocytes, and some mononuclear macrophages. The myometrium was moderately edematous. These changes are shown (fig. 17–27).

Placentas in advanced stages of vibriosis had changes that were described for early stages of vibriosis in addition to other alterations. In these placentas, most changes were limited to the hilar zone. Some septums of each sheep had arteriolitis. Endothelial cells were necrotic and degenerated, and leukocytes infiltrated the vessel wall. Bacteria were not discernible in the vessel. Both arterioles and arterial capillaries were affected.

From foci of bacterial growth, especially in hematomas, *V. fetus* had penetrated chorionic villi. Many epithelial cells of primary villi contained within the cytoplasm large numbers of the granular form of *V. fetus*. Such cells were enlarged and basophilic. Frequently, the large number of bacteria in the cytoplasm obscured the nucleus and other cellular structures. Parasitized epithelial cells were unevenly distributed throughout the hilar zone. In foci of infection, affected cells usually were close together but not necessarily contiguous. In ewes with apparent imminent abortion, much of the chorionic epithelium in the hilar zone and, in some cases, throughout most of the placentome, was necrosed and detached from the basement membrane of the villi. The desquamated epithelial cells and cellular debris accumulated in the

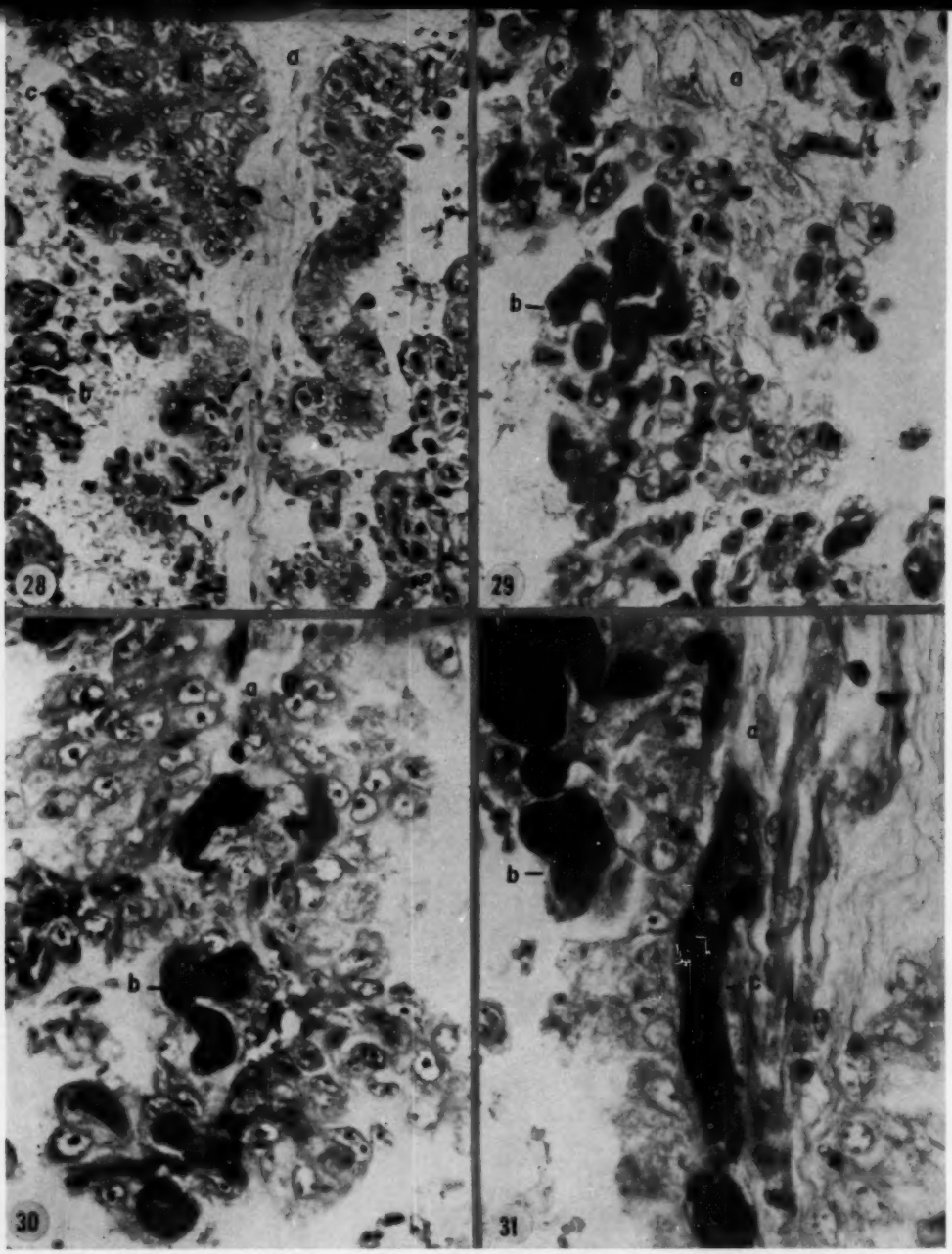

Fig. 28—Placentome in advanced vibriosis from experimental sheep in fifth month of gestation. (a) Primary villus; (b) septum; and (c) colonies of Vibrio fetus in cytoplasm of chorionic epithelium. H & E stain; x 172.

Fig. 29—Chorionic villus in advanced vibriosis of sheep in fifth month of gestation. (a) Primary villus and (b) colonies of Vibrio fetus in chorionic epithelium. Phloxine methylene blue stain; x 504.

Fig. 30—Chorionic villus in advanced vibriosis of experimental sheep in fifth month of gestation. (a) Base of villus and (b) colony of Vibrio fetus in epithelial cell and capillary. Phloxine methylene blue stain; x 540.

Fig. 31—Chorionic villus in advanced vibriosis of experimental sheep in fifth month of gestation. (a) Stroma of villus; (b) colony of Vibrio fetus in epithelial cell; and (c) colony of Vibrio fetus in endothelial cells and lumen of capillary. Giemsa stain; x 544.

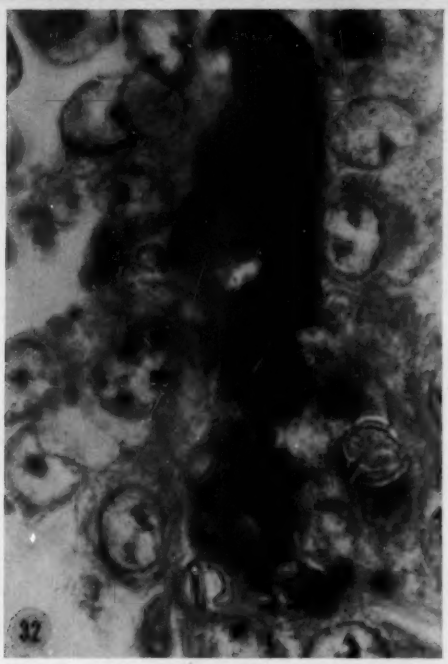

Fig. 32—Colony of Vibrio fetus in capillary of a villus from advanced vibriosis in experimental sheep in fifth month of gestation. Giemsa stain; x 1,260.

space between chorionic villi and endometrial septums. This changed the normal ratio of approximately equal parts of chorionic and endometrial tissue within the placentome to a predominance of septums. Beginning separation of chorion from endometrium was evident in some placentomes.

In foci of intraepithelial colonies of bacteria, *V. fetus* had penetrated adjacent mesodermal portions of the villi. Capillaries, either intraepithelial or subepithelial, contained colonies of *V. fetus*. The cytoplasm of endothelial cells appeared to be penetrated and, in some cases, the vessel lumens were partially or completely occluded. The colonies assumed tortuous outlines of capillaries. In areas of heavy infection, *V. fetus* also invaded chorionic connective tissue. Edema was extensive in the chorion at the cotyledonary hilus. In many areas, the myometrium was severely edematous. In smears of cotyledons, there were abundant free *V. fetus* with typical morphology and many epithelial cells con-

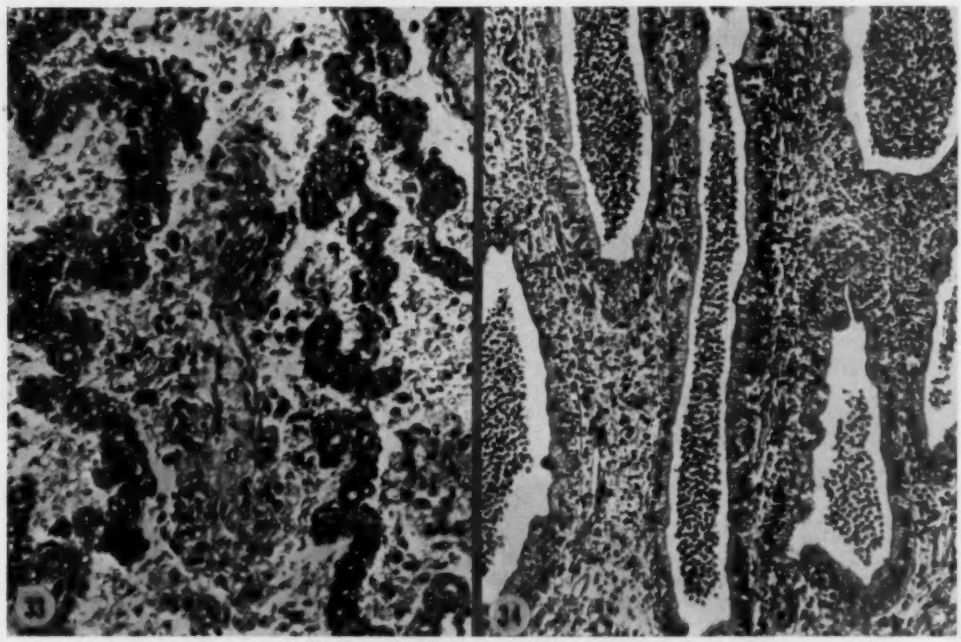

Fig. 33—Placentome in advanced vibriosis from experimental sheep in fifth month of gestation. (a) Villus with detached and necrotic epithelial cells, (b) septum; and (c) debris from necrotic cells. H & E stain; x 136.

Fig. 34—Interplacentomal endometritis in experimental sheep affected with advanced vibriosis. H & E stain; x 136.

taining *V. fetus* in the cytoplasm. Some mononuclear macrophages also contained ingested *V. fetus*. These changes are shown (fig. 28–34). *Vibrio fetus* was isolated in cultures prepared from the cotyledons and from fetuses. The histopathologic changes are listed (table 3).

EXPERIMENT 2

Normal Placentas.—In 5 fetal placentas from ewes having normal parturition, there were dark red cotyledons and thin, translucent, intercotyledonary tissue. Decomposition was not advanced.

Microscopically, autolysis and decomposition were minimal. Endometrial tissue, except for an occasional fragment of septum, was absent. Chorionic villi were small from contraction and were covered with epithelium. Chorionic blood vessels were hyperemic. Plant tissue and other contaminants were often present.

Vibriosis-Affected Placentas.—Membranes from ewes which aborted as a result of vibriosis were seriously decomposed. Often, the membranes had focal edema and hemorrhages (fig. 36).

Histopathologic changes in placentas from ewes with spontaneous vibriosis were similar to changes in placentas of animals inoculated experimentally with pathogenic *V. fetus*. Of the 36 placentas, 31 (86%), although in advanced stages of decomposition, contained intraepithelial and intracapillary colonies of *V. fetus*. These were readily discernible (fig. 37, 38). Edema of connective tissue was often prominent. Most smears prepared from unfixed membranes contained organisms with morphologic characteristics of *V. fetus* (fig. 39). The other 5 (14%) placentas had severe inflammation of the chorion with infiltration by large numbers of leukocytes, especially neutrophils. Hyperemia and edema were prominent. Intraepithelial and intracapillary colonies of *V. fetus* were not identifiable.

Uteri from ewes which died from vibriosis contained macerated fetuses. Metritis with foci of necrosis through the entire uterine wall caused peritonitis and, in some cases, discharge of uterine contents into the peritoneal cavity (fig. 40).

DISCUSSION

The gross changes of vibriosis-affected sheep observed in this study were in agreement with those described by McFadyean and Stockman. Changes were limited to the uterus and its contents. The uterine wall was edematous. Placentomes were swollen and soft in consistency. Cotyledons separated readily from caruncles and were pale. Some exudate mixed with blood was located between chorion and endometrium at the interplacentomal areas.

The study of entire and intact placentas from sheep affected experimentally with vibriosis during the fifth month of gestation, and the study of fetal membranes from aborting ewes infected naturally, revealed similar lesions in several stages of development. These changes suggested the following pathogenesis of vibriosis: Maternal bacteremia resulting from *V. fetus* caused localization of infection in the hilar zone of placentomes. Many septums developed arteriolitis with necrosis of the vessel wall. Some affected arterioles and capillaries became thrombosed. From maternal blood, *V. fetus* penetrated through vessels in the degenerated septal extremities from which originated hematomas, gaining access to the maternal extravasations between chorion and septums. Organisms may also have penetrated through the walls of maternal arterioles injured by the disease. In the hematomas in lacunas, *V. fetus* proliferated and stimulated the accumulation of leukocytes in injured septums as well as in hematomas. Some organisms actively penetrated or were passively phagocytized by

Fig. 35—Vulvitis and infected discharge in sheep following abortion from vibriosis. x 0.8.

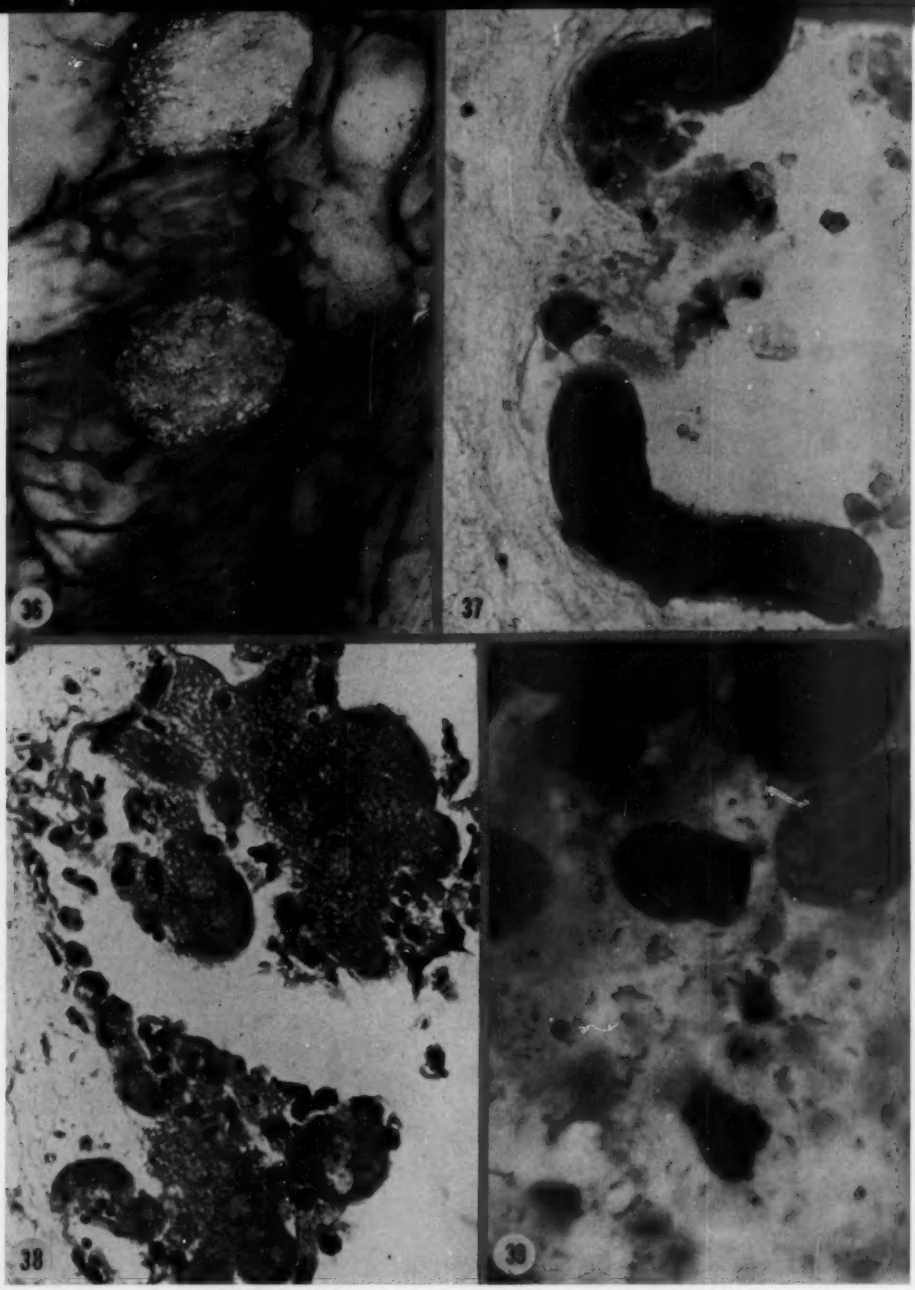

Fig. 36—Chorion from sheep which aborted from natural vibriosis. Cotyledons are pale and intercotyledonary tissue is edematous. x 1.0.

Fig. 37—Chorion from sheep which aborted from natural vibriosis. Epithelium has been destroyed by decomposition. Two capillaries are filled with colonies of Vibrio fetus. H & E stain; x 1,506.

Fig. 38—Chorion from sheep which aborted from natural vibriosis. Epithelial cells contain colonies of Vibrio fetus. H & E stain; x 506.

Fig. 39—Vibrio fetus in smear of vulvar exudate following abortion from vibriosis. Giemsa stain; x 1,200.

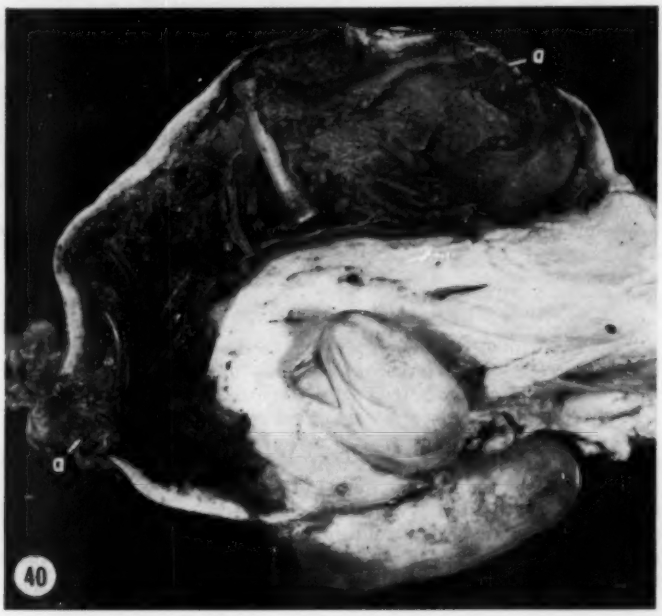

Fig. 40—Dissected uterus from sheep which died from vibriosis. Uterine horn contained macerated fetus. Infected uterine contents discharged into the peritoneal cavity through foci of necrosis (a).

chorionic epithelium. Intracytoplasmic organisms became abundant along with enlargement of affected epithelial cells and obscurity of cell details. In multiple foci, many chorionic epithelial cells became parasitized. From the infected epithelial cells, or possibly directly from the hematomas, the organisms penetrated chorionic capillaries adjacent to or within epithelium. Capillary endothelial cells also were penetrated, and the cytoplasm became filled with organisms. Some capillary lumens were occluded with colonies of *V. fetus*. From these vessels, organisms had free access to fetal circulation and fetal liver. Connective tissue of the chorion, and especially of chorionic villi, became edematous. In areas near infected capillaries, *V. fetus* penetrated the connective tissue. In advanced stages of the disease, most chorionic epithelium of the villi desquamated into the space between septums and villi, and the villi readily separated from septums. The fetus died because of bacteremia and possibly because of toxemia from extensive infection within the chorion. Fetal hypoxia may have resulted from occlusion of many capillaries within the chorion and from thrombosis of some arterioles and capillaries of maternal septums. Following fetal death, infected fetus and membranes decomposed and autolyzed rapidly. Uterine contents infected with *V. fetus* were expelled, and the environment became contaminated (fig. 35).

The manner by which chorionic epithelial cells were penetrated is equivocal: organisms may have actively penetrated passive cells and proliferated within the cytoplasm to form colonies of organisms, or epithelial cells may have actively phagocytized the organisms, since chorionic epithelium of the arcades phagocytized fragments of, as well as entire, erythrocytes. After being phagocytized, the organisms may have then proliferated to form intracellular colonies. In advanced stages, when abortion was imminent, most of the chorionic epithelium of the villi desquamated into the space between septums and villi. This may have resulted from toxic metabolites from organisms within hematomas and within epithelial cells. Hypoxia from circulatory interference in septums may also have contributed to the cause of desquamation.

Extensive edema and pale discoloration of cotyledons readily differentiated the vibriosis-affected membranes from normal membranes. Frequently, at abortion, gross changes were obscured by decomposition.

The diagnosis of vibriosis by practicing veterinarians may be established with high accuracy by careful examination of aborted fetal membranes for gross lesions and by the examination of Giemsa-stained smears from pale cotyledons for *V. fetus*. Touch smears stained with Giemsa or Wright's stain differentiated the characteristic morphology of *V. fetus* (fig. 39). The feasibility of this means of diagnosis has been demonstrated by some practicing veterinarians.

SUMMARY

Two experiments were conducted to determine gross and microscopic lesions of placentas of sheep affected with vibriosis. In experiment 1, 14 sheep were inoculated intravenously with pathogenic *Vibrio fetus* at the beginning of the fifth month of gestation. Five similar sheep were not inoculated. The 19 animals were euthanatized 4 to 8 days following inoculation and placentas were obtained for study. In experiment 2, 36 fetal membranes were obtained from ewes which aborted from vibriosis, and changes in the membranes of these spontaneously infected animals were compared with the changes resulting from experimental vibriosis.

Placental changes due to experimental and spontaneous vibriosis were similar. In advanced stages of the disease, the uterus was edematous, especially at the lesser curvature of the horn. Placentomes were swollen, and the chorion contained many foci of edema. Infected cotyledons separated easily from caruncles. The cotyledons were abnormally pale.

Histopathologically, the changes were concentrated in the hilar zone of placentomes. Septums had arteriolitis and some thrombosis. Bacteria were abundant in hematomas of the lacunas. In advanced stages of the disease, colonies of *V. fetus* were found in the cytoplasm of many chorionic epithelial cells and in the endothelium and lumens of adjacent chorionic capillaries. Chorionic epithelium of the villi desquamated into the space between septums and villi.

Consideration of the gross lesions of infected placentas and the demonstration of *V. fetus* in stained smears from cotyledons provided an easy and accurate basis for field diagnosis.

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SUMMARIO IN INTERLINGUA

Pathologia Placental in Oves con Vibriosis

Duo experimentos esseva executate pro determinar le lesiones macro- e microscopic in placentas de oves afficite de vibriosis. Dece-quatro oves recipeva inoculationes intravenose de pathogene *Vibrio fetus* al comenciamento del quinte mense de gestation. Cinque comparabile oves non recipeva inoculationes. Le 19 animales esseva euthanatisate 4 a 8 dies post le inoculation, e lor placentas esseva obtenite pro le studio. Trenta-sex membranas fetal esseva obtenite ab oves que abortava a causa de vibriosis, e le alterationes in le membranas de iste spontanee-mente inficite animales esseva comparate con le alterationes resultante de vibriosis experimental.

Le alterationes placental causate per vibriosis experimental e illos causate per vibriosis spontanee esseva simile. In avantiate stadios del morbo, le utero esseva edematose, special-mente in le curvatura minor del corno. Placentomas esseva tumide, e le chorion contineva multe focos de edema. Inficite cotyledones se separava facilemente ab le carunculas. Le cotyledones esseva anormalmente pallide.

Ab le puncto de vista histopathologic, le alterationes esseva concentrate in le zona hilar de placentomas. Septos habeva arteriolitis, e certes habeva thrombosis. Bacterios esseva

abundante in hematomas del lacunas. In avantiate stadios del morbo, colonias de *V. fetus* esseva trovate in le cytoplasma de multe cellulas de epithelio chorionic e in le endothelio e le passage de adjacente capillares chorionic. Epithelio de villos se desquamava ad in le spatio inter septos e villos.

Le scrutinio del lesiones macroscopic in inficite placentas e le demonstration de *V. fetus* in tincturate frottis ab cotyledones provideva un facile e accurate base pro diagnoses in le campo.

Studies on Immunity to *Haemonchus contortus* Infection—Double Vaccination of Sheep with Irradiated Larvae

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IN AN EARLIER REPORT [1] we described the successful immunization of sheep against *Haemonchus contortus* by using as a vaccine infective larvae which had been partially inactivated by x-rays. A range of roentgen dosages was explored and it was found that larvae exposed to 40,000 and 60,000 roentgens produced a good immunity to challenge with 8,000 larvae. Previous experience with *Dictyocaulus viviparus* had shown [2] that 2 doses of a similar vaccine induced a much greater resistance to reinfection than did a single dose. Since field infections in lambs can be much greater than 8,000 *H. contortus* larvae, it was decided to apply the double-dose method and to subject the lambs to higher challenge levels.

TABLE 1—Experimental Plan of Vaccination of Sheep with Irradiated Haemonchus contortus Larvae Followed by Challenge with Normal Larvae

Group	Day 0	Day 35	Day 64	Day 114
1	10,000 irradiated larvae	10,000 irradiated larvae	10,000 normal larvae	Killed
2	10,000 irradiated larvae	10,000 irradiated larvae	50,000 normal larvae	Killed
3	.....	.....	10,000 normal larvae	Killed
4	.....	.....	50,000 normal larvae	Killed

MATERIALS AND METHODS

Twenty-two newborn lambs were bought and raised worm-free. The method of feeding and the parasitologic and irradiation techniques were described in an earlier report.[1] The lambs were 8 months old at the start of the experiment.

The lambs were allotted to 4 groups. There were 5 lambs each in groups 1 and 2 (principals), and 6 lambs each in groups 3 and 4 (controls). On day zero, the lambs in groups 1 and 2 were given orally 10,000 third-stage larvae which had been irradiated the same day with 40,000 roentgens. On day 35, this was repeated. On day 64, the immunity of the lambs of groups 1 and 3 was challenged with 10,000 normal infective larvae and that of the lambs in groups 2 and 4 with 50,000 normal larvae. All of the animals were killed on day 114, when abomasal worm counts were made. Fecal egg counts by the McMaster method were carried out at approximately weekly intervals throughout the experiment. The design of the experiment is summarized (table 1).

RESULTS

The fecal egg output and abomasal worm burdens of the lambs are shown (tables 2–5). The group mean egg counts of the controls followed the expected pattern: The control lambs given 10,000 larvae reached a mean peak of excretion of 3,225 eggs per gram 28 days (day 92) after challenge; those given 50,000 larvae reached 10,975 eggs per gram on the same day; the corresponding counts of the principals were 0 and 20, respectively. The abomasal worm burdens paralleled these results. The principals given the lower challenge dose (group 1) had a mean of 25.8 worms as compared with 1,942 in the controls (group 3); the mean counts for lambs given a challenge dose of 50,000 larvae were 5.8 in the principals and 2,455 in the controls (table 6).

DISCUSSION

The results indicated that by giving 2 doses of vaccine a considerable degree of resistance to high challenge doses was obtained. This would probably be sufficient to protect lambs against field infections, but a factor still to be explored before assessing the field possibilities of a vaccine is the duration of the immunity.

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TABLE 2—Parasitologic Findings in Lambs of Group 1, Vaccinated with Irradiated Larvae and Given a Challenge Dose of 10,000 Normal Larvae of Haemonchus contortus

Lamb No.	Eggs per gram of feces on day								No. of worms at necropsy
	64*	66	78	85	92	99	106	113	
1	....	0	0	0	0	0	0	0	0
2	....	50	0	0	0	0	0	0	24
3	....	0	0	0	0	0	0	0	27
4	....	0	0	0	0	0	0	0	12
5	....	0	0	0	0	0	100	50	66
Mean	....	10	0	0	0	0	20	10	26

* Point of challenge.

TABLE 3—Parasitologic Findings in Lambs of Group 2, Vaccinated with Irradiated Larvae and Given a Challenge Dose of 50,000 Normal Larvae of Haemonchus contortus

Lamb No.	Eggs per gram of feces on day								No. of worms at necropsy
	64*	66	78	85	92	99	106	113	
6	....	0	0	0	50	50	0	0	14
7	....	0	100	0	0	0	0	0	11
8	....	0	50	0	0	0	0	0	0
9	....	0	50	0	0	0	0	0	0
10	....	0	0	0	50	50	0	20	4
Mean	....	0	40	0	20	20	0	4	6

* Point of challenge.

TABLE 4—Parasitologic Findings in Unvaccinated Lambs of Group 3, Given a Challenge Dose of 10,000 Normal Larvae of Haemonchus contortus

Lamb No.	Eggs per gram of feces on day								No. of worms at necropsy
	64*	66	78	85	92	99	106	113	
11	....	0	0	0	2,450	1,950	2,350	1,450	827
12	....	0	0	50	5,400	1,300	2,400	500	1,775
13	....	0	0	850	4,550	6,800	6,900	7,800	3,880
14	....	0	0	0	2,200	2,300	2,600	2,500	2,680
15	....	0	0	50	3,350	3,750	1,600	350	1,346
16	....	0	0	0	1,400	1,650	1,200	1,000	1,145
Mean	....	0	0	158	3,225	2,958	2,841	2,267	1,942

* Point of challenge.

TABLE 5—Parasitologic Findings in Unvaccinated Lambs of Group 4, Given a Challenge Dose of 50,000 Normal Larvae of Haemonchus contortus

Lamb No.	Eggs per gram of feces on day								No. of worms at necropsy
	64*	66	78	85	92	99	106	113	
17	....	0	0	0	7,050	7,250	9,750	12,800	2,740
18	....	0	0	600	23,250	13,300	6,050	10,600	4,112
19	....	0	0	0	5,550	5,000	6,650	550	1,554
20	....	0	0	2,900	12,900	10,350	9,150	2,750	1,367
21	....	0	0	200	9,900	550	200	350	630
22	....	0	0	650	7,200	4,650	7,050	10,350	4,324
Mean	....	0	0	725	10,975	6,850	6,475	6,233	2,455

* Point of challenge.

TABLE 6—The Peak Mean Fecal Egg Counts per Gram and the Group Mean Abomasal Worm Counts for Lambs Vaccinated with Irradiated Haemonchus contortus Larvae and Later Given a Challenge Dose of Normal Larvae

Group No.	Peak mean e.p.g.	Mean abomasal worm counts
1	20.0	25.8
2	40.0	5.8
3 (controls)	3,225.0	1,942.0
4 (controls)	10,975.0	2,455.0

SUMMARY

A vaccine was prepared by subjecting infective larvae of *Haemonchus contortus* to 40,000 roentgens of x-radiation. When 2 doses of vaccine (each containing 10,000 larvae) were given to lambs, a degree of immunity sufficient to withstand a challenge with 50,000 normal larvae was produced.

References

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[2] Jarrett, W. F. H., Jennings, F. W., McIntyre, W. I. M., Mulligan, W., Sharp, N. C. C., and Urquhart, G. M.: Immunological Studies on *Dictyocaulus viviparus* Infection in Calves—Double Vaccination with Irradiated Larvae. Am. J. Vet. Res., 20, (May, 1959): 522–526.

SUMMARIO IN INTERLINGUA

Studios in Re le Immunitate Contra Infection per *Haemonchus contorsus* —Duple Vaccination de Oves con Irradiate Larvas

Un vaccino esseva preparate per subjicer infective larvas de *Haemonchus contorsus* a 40.000 r de radiation X. Quando duo doses de vaccino (continente 10.000 larvas cata un) esseva administrate a agnos, un grado de immunitate esseva producite que sufficeva pro resister a un provocation con 50.000 larvas normal.

Studies on the Pathology of Johne's Disease in Sheep
III. Pathologic Changes in Sheep with Naturally Occurring Infections

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JOHNE'S DISEASE (paratuberculosis) in sheep is of world-wide distribution, causing a nonspectacular but heavy aggregate mortality. Variations in the pathologic lesions have been reported frequently in the literature, and it appears that descriptions pertained to sheep in the advanced stage of the disease. Little is known of the early lesions and their relationship to the progress of this disease in sheep.

The present investigations, designed to study the relationships of the clinical manifestations in reactor sheep in various stages of the disease, included hematologic studies, studies of pathologic changes, and bacteriologic examinations. It was hoped that the histopathologic studies might indicate the pathogenesis of this malady.

MATERIALS AND METHODS

Material was obtained from 19 Bikaneri sheep (4 rams and 15 ewes) belonging to the District Dairy Demonstration Farm at Mathura, India. These sheep gave positive reactions to allergy tests conducted in 1955–1956, using johnin and avian tuberculin intradermally. Two lambs from ewes in this group were also utilized for this study. Additional material was obtained from 2 lambs from sheep 300 and 1 lamb from sheep 315, born during the course of observation. They were weaned immediately after birth and were maintained on goats' milk. These lambs were used to study congenital infection. Material for this purpose was also collected from the fetus of a sheep (262) which was killed.

In an attempt to study the correlation, if any, between the extent of gross and histopathologic lesions, number of organisms found, and physical condition of the animals, the 19 sheep were allotted to 3 groups on the basis of clinical signs and body condition. Group I sheep (4 rams and 2 ewes, *see* table 1) were extremely weak and emaciated. Group II sheep (1 ram and 6 ewes, *see* table 2) were weak but not emaciated. Group III sheep (1 ram and 5 ewes, *see* table 3) were apparently healthy.

With the exception of 4 sheep (233, 187, 220, and 273) that died of the disease, the rest, including 5 lambs, were killed. The viscera were examined immediately for gross lesions. Smears for bacteriologic examination were prepared from the ileocecal valve, ileum, jejunum, duodenum, and mesenteric and ileocecal lymph nodes. Material for histopathologic examination was collected from the abomasum; various levels of the intestinal tract and contiguous lymph nodes; mediastinal, parotid, supramammary, hepatic, and pancreatic lymph nodes; tonsils; thoracic duct; spleen; liver; lungs; kidneys; adrenals; corneas; heart; pericardium; loose fascia; skin; uterus; udder; pancreas; testicles; fetal membranes; cotyledons; and umbilical cord. The gross and histopathologic lesions are described only for those tissues which were found to have significant lesions. The material was fixed in 10 per cent formol solution. Paraffin-embedded tissue sections were cut at 4 to 5 μ and stained with hematoxylin and eosin (alcoholic method). For demonstration of acid-fast bacilli, the deparaffinized sections were passed through the alcohol series to water and treated for a few minutes with a 1 per cent aqueous ammonia solution. They were stained by a modified Ziehl-Neelsen technique, using hematoxylin for the nuclear stain and a saturated solution of tartrazine as a counterstain.[12]

The isolation of *Mycobacterium paratuberculosis* was accomplished from an affected enlarged mesenteric lymph node on Dunkin's medium.

RESULTS

GROUP I

Clinical and pathologic observations on the 6 group I sheep revealed that they were in an advanced stage of the disease.

Gross Pathologic Changes.—At necropsy, the cadavers were characterized by extreme emaciation, absence of fatty deposits, and skeletal muscle atrophy. The fat depots were replaced by myxomatous tissue which sometimes contained chalky deposits, indicating fat necrosis. In 2 sheep, there was edematous swelling of the dependent parts, particularly of the submaxillary region.

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The authors thank C. V. G. Choudary, principal, Utter Pradesh College of Veterinary Science and Animal Husbandry, Mathura, for the facilities provided in conducting the work.

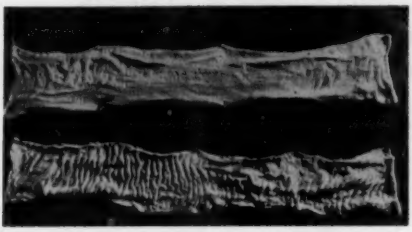

Fig. 1—Corrugation of the mucous membrane in segments of ileum (group I).

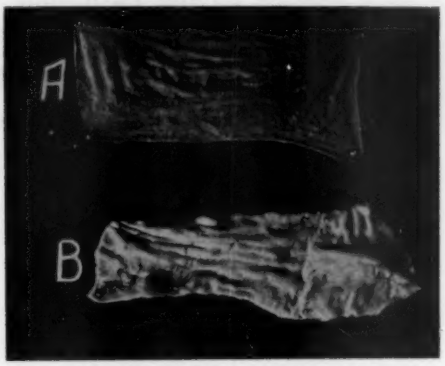

Fig. 2—Segments of ileum: (A) congestion without any distinct thickness; (B) fissures in the mucosa with granular thickening (group I).

The fluid in the peritoneal, pericardial, and pleural cavities was excessive. Intestinal lesions varied from extremely congested patches, involving considerable portions of the intestinal mucosa, to a thickened or corrugated appearance of the mucosa. The relationship of the type of lesions in the intestinal wall to the presence of acid-fast organisms in the smears is shown (table 1).

The contiguous lymph nodes of the mesentery and the ileocecal valve were enlarged, and a milky fluid oozed from the cut surface. The mesenteric lymph nodes of sheep 140 had nodular outgrowths (fig. 3). In 2 sheep (219 and 273), a few of the mesenteric lymph nodes had caseous and calcified foci. In all 6 sheep, acid-fast organisms could easily be seen in smears from mesenteric lymph nodes and the lymph nodes related to the ileocecal valve. In addition to these lesions, there was bilateral opacity of the corneas in 1 of the sheep.

Histopathologic Changes.—The tissue reaction of the mucosa of the ileum was granulomatous inflammation. The changes involved all but the muscular layers. In most of the sheep, the epithelium was desquamated; coagulative necrosis affected the superficial portion of the mucosa. The unusual vascularity and hemorrhagic spots in the mucosa were striking features. The lamina propria was heavily infiltrated with mononuclear phagocytic cells. This increased cellularity caused distention and distortion of the villi. In some sections, the villi had undergone degenerative changes and contained extracellular acid-fast rods. The extensive cellular infiltration further affected the replacement of crypts and resulted in their actual reduction, as evidenced by atrophy and degenerative changes (fig. 4, 5).

The proliferating cells which infiltrated the lamina propria of the mucosa were

TABLE 1—Relationship of Lesions in the Intestinal Wall to the Presence of Acid-Fast Organisms in the Smears (Group I)

Sheep No.	Type of lesion at different level of the intestinal wall			Smear examination for acid-fast rods	
	Ileum	Ileocecal valve	Duodenum	Ileum	Ileocecal valve
148	Congestion and corrugations with pinkish, slimy exudate (fig. 1).	Congested and edematous with slimy exudate.	Corrugations up to 2 ft. from pyloric end.	+ (heavy)	+ (heavy)
187	Thickened and corrugated with slimy mucus.	Congestion extending over cecum around the valve.	NS*	+ (heavy)	+ (heavy)
233	Excessive congestion of 30 ft. of ileum with slimy, pale exudate (fig. 2A).	Congested.	NS	+ (heavy)	+ (heavy)
219	Thickening with corrugations and slimy mucous exudate.	Thickened and congested.	NS	+ (heavy)	+ (heavy)
273	Terminal portion extremely congested.	Congested.	NS	+ (heavy)	+ (heavy)
220	Congestion with slimy exudate and no thickening.	Congested.	NS	+ (heavy)	+ (heavy)

* NS = no significant lesions.

Fig. 3—Mesenteric lymph nodes: (AA') enlargement with nodular thickening; (B) enlargement with whitish opaque cortex.

macrophages and epithelioid and giant cells (both Langhan's and foreign-body types). Plasma cells in increased proportion and lymphocytes and eosinophils in small numbers were present, also. The occurrence of these cells in the lamina propria in the same section varied from focal aggregates surrounded by lymphocytes to diffuse areas of epithelioid cell aggregation and even to the formation of synclinal masses.

Examination of sections stained by the Ziehl-Neelsen method revealed acid-fast organisms phagocytized by the macrophages, epithelioid cells, and giant cells (fig. 6, 7). The cytoplasm of these cells was heavily packed with acid-fast rods, with no apparent damage to the nucleus despite the fact that, in most cases, the nucleus was obscured by the heavy number of these organisms (fig. 7). Macrophages containing rods were seen in the lumens of the crypts, and they further infiltrated the muscularis mucosa and submucosa. In some sections, the macrophages had entered the submucosa after damaging the muscularis mucosa (fig. 8). The submucosa contained congested capillaries. Infiltrating macrophages containing rods obliterated the lumens of lymphatics and blood vessels and formed thrombi.

In one vein, there was a distinct thrombus (fig. 9) composed of an acidophilic mass with degenerated cellular nuclei and erythrocytes. The outer zone of the thrombus consisted mostly of macrophages with intracellular acid-fast rods in varying stages of degeneration (fig. 10). The central portion contained many acid-fast granules. The internal wall had thickened and was infiltrated with leukocytes.

The changes in the duodenal mucosa were, to some extent, similar but the infiltration of cells in the lamina propria was not so intense and the acid-fast rods were also scanty.

The most common histopathologic change in the lymph nodes was infiltration of subcapsular, cortical, and medullary sinuses by macrophages containing acid-fast rods, along with epithelioid and giant cells (fig. 11–13). In the germinal centers, there was marked accumulation of macrophages, some of which had assumed giant size (fig. 14). In many sections, the reaction was extremely diffuse and symplasm stages were markedly distinct. The reticular tissue in some cases

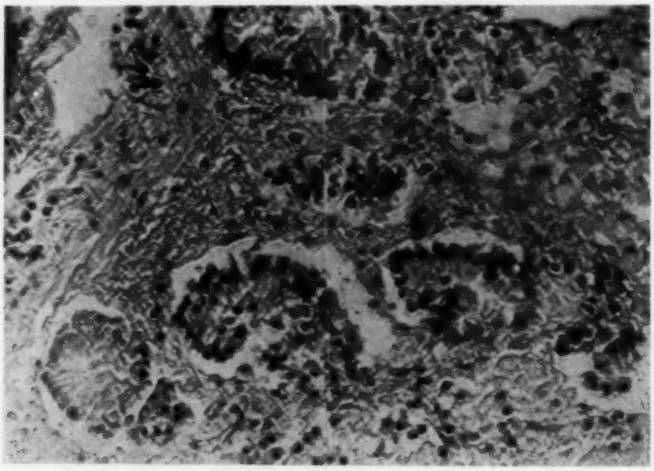

Fig. 4—Desquamation of lining epithelium and necrosis of crypts in the ileum (group I). H & E stain; x 310.

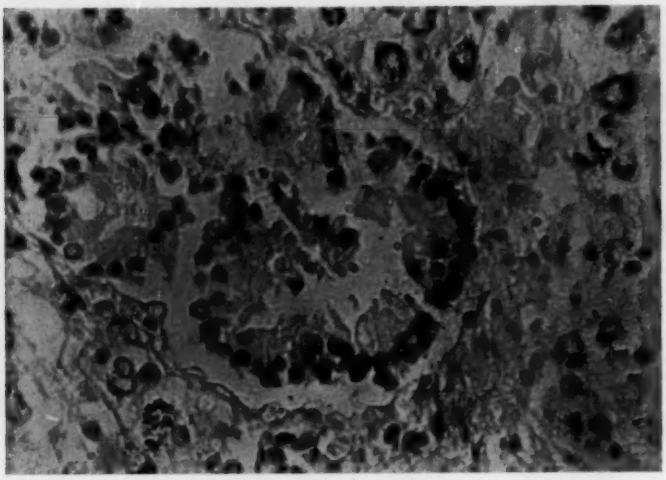

Fig. 5—Desquamation and degeneration of cells in crypts of the ileum, surrounded with macrophages containing acid-fast rods (group I). Carbol-fuchsin and hematoxylin; x 450.

was distended and edematous. In a few sections, exhaustion of the lymphoid tissue was also prominent. Caseous and calcified foci were seen in 3 sheep (fig. 15). These lesions varied from necrotic foci, surrounded by a zone of mononuclear phagocytic cells containing acid-fast rods, to complete encapsulation of the caseous and calcified foci which also contained acid-fast rods. However, acid-fast organisms could not be demonstrated in one mesenteric lymph node having concentric rings of calcified and caseous material. Granulomatous inflammation was found in the tonsils and in the parotid, mediastinal (fig. 16), hepatic, and colic lymph nodes along with acid-fast organisms in all but the mediastinal lymph nodes.

Intracellular acid-fast organisms were found in the macrophages that had infiltrated the epicardium along with lymphocytes and a few giant cells. In liver and lung tissues, acid-fast bacilli but no significant microscopic changes could be seen. In the corneal epithelium, there was keratinization, excessive cellular infiltration by

TABLE 2—Relationship of Lesions to the Presence of Organisms in the Intestinal Smears (Group II)

Sheep No.	Type of lesion at different level of the intestinal wall			Smear examination for acid-fast rods	
	Ileum	Ileocecal valve	Duodenum	Ileum	Ileocecal valve
X	Congested at places.	Congested.	NS*	—	—
262	Congested mainly about 1' of the terminal portion of ileum.	Congested.	NS	—	—
203	Congestion in bands of 2 mm. width. Mucosa covered with slimy mucus.	Slightly congested.	NS	+	+
299	Congestion mainly near the terminal portion of ileum.	Congested.	NS	—	—
274	Slight granular thickening with distinct corrugation; longitudinal fissures in mucosa (fig. 2B).	Slightly congested.	NS	—	—
360	Congested and thickened at places with creamish, slimy exudate over mucosa.	Slightly congested.	NS	—	—
239	Congested and thickened with nodular wormlike lesions of the bowel.	Congested with nodular, wormlike lesion.	NS	—	—

* NS = no significant lesions.

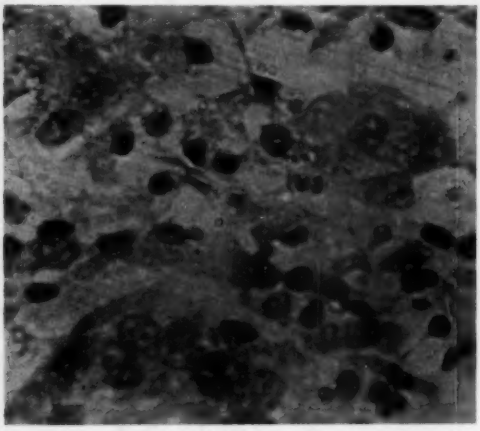

Fig. 6—Giant cells and macrophages containing acid-fast rods in the lamina propria of the ileum (group I). Carbol-fuchsin and hematoxylin; x 900.

neutrophils and some mononuclear cells, congestion of episcleral arterial vessels, and a zone of hyalinization with necrosis.

GROUP II

Gross Pathologic Changes.—Five of the 7 sheep in group II were not emaciated; emaciation in sheep 203 and 239 was not as marked as that observed in group I sheep and no significant reduction of fat in the fat depots was found. The gross changes in the intestinal wall and the results of examination for acid-fast organisms are shown (table 2).

The mesenteric and ileocecal lymph nodes underwent comparatively little change. However, the mesenteric lymph nodes were edematous in 3 sheep and contained caseous and calcified foci with greenish matter in 2 sheep. No acid-fast organisms were found in the smears of lymph nodes in this group.

Histopathologic Changes.—Cellular infiltration was observed in the mucosa of the ileum with slight extension in a few cases into the submucosa and mucosa muscularis. Although the infiltrative changes caused distention of the villi, there was little or no desquamation of the epithelial lining. Hyperemic changes were prominent in the mucosa and submucosa in most cases. The cellular infiltration consisted of plasma cells and some macrophages, lymphocytes, and eosinophils. Focal aggregation of epithelioid cells in the villi and a few giant cells were present in some tissue sections. A few macrophages had undergone division and contained 1 or 2 acid-fast rods. The presence of acid-fast rods was scanty in the tissue sections of ileum and ileocecal valve, except in 1 sheep in which moderate numbers of organisms were found, both intra- and extracellularly, in the macrophages. In 2 sheep, acid-fast granules and acid-fast rods were observed in macrophages.

The mesenteric and ileocecal lymph nodes had moderate accumulations of macrophages in the germinal centers (fig. 17), and the capsular and cortical sinusoids were infiltrated in places by macrophages and plasma cells. A few epithelioid and giant cells could be seen, also. Some of the mesenteric and ileocecal lymph nodes had a few acid-fast rods in the macrophages.

GROUP III

Gross Pathologic Changes.—Necropsy of the 6 sheep in group III revealed no noticeable changes comparable to the lesions of paratuberculosis. The fat depots contained almost normal amounts of fat and the skeletal muscles had no significant changes. The lesions of the intestinal tract and the results of smear examinations for acid-fast bacilli are listed (table 3).

No significant gross lesions could be observed in lymph nodes, except in 2 sheep in which the mesenteric lymph nodes were found to be somewhat enlarged and juicy.

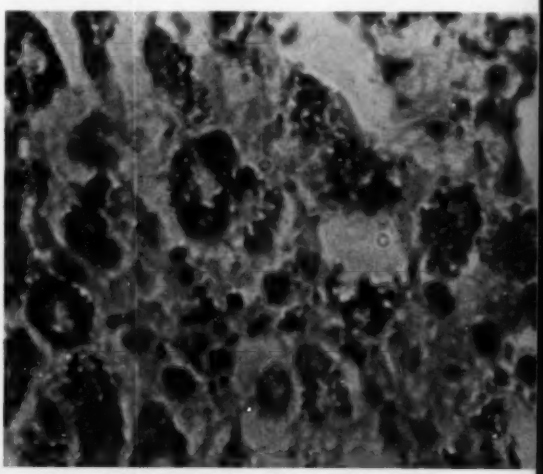

Fig. 7—Acid-fast organisms in macrophages in lower third of mucosa of ileum (group I). Carbol-fuchsin and hematoxylin; x 1,260.

TABLE 3—Relationship of Lesions to the Presence of Organisms in Intestinal Smears (Group III)

Sheep No.	Type of lesion at different level of the intestinal wall			Smear examination for acid-fast rods	
	Ileum	Ileocecal valve	Duodenum	Ileum	Ileocecal valve
X[2]	Thickened and congested at places.	Congested	NS*	—	—
308	Congested at places.	—	NS	—	—
241	Congested, thickened with smaller petechial areas at places only.	—	NS	—	—
271	Slightly congested and thickened at places.	—	NS	—	—
315	Congested at places with yellowish pink exudate over the mucosa.	—	NS	—	only a few rods
300	Congested at places.	—	NS	—	—

* NS = no significant lesions.

Histopathologic Changes.—Cellular infiltration in the ileum consisted of lymphocytes with some plasma cells, eosinophils, and focal aggregates of macrophages (fig. 18). There was no cellular infiltration in the submucosa. Acid-fast rods could not be seen in tissue sections of the ileum, except for a few noticed in tissues of sheep 271.

There was some infiltration of macrophages in the germinal centers of the mesenteric lymph nodes. Significant changes were not observed in the sinusoids, except for some edema, and acid-fast organisms were not demonstrated in tissue sections of lymph nodes.

DISCUSSION

Pathologic changes in sheep with paratuberculosis have been reported by several workers. However, there appears to be little conformity in the lesions described in their studies. The present investigation, correlating the clinical manifestations with gross and histopathologic findings, indicates the variability of lesions at different stages of this disease. In essence, however, the tissue reactions conform to granulomatous inflammation.

The occurrence of edematous swelling in the dependent parts, particularly in the submaxillary region, and excess fluid in serous cavities of most group I sheep conforms to the observations of Howarth [11] and Nanda and Singh,[20] who related this condition to advanced stages of the disease. These features of the disease can be related to its wasting and chronic nature, leading to cachexia. This might be the result of

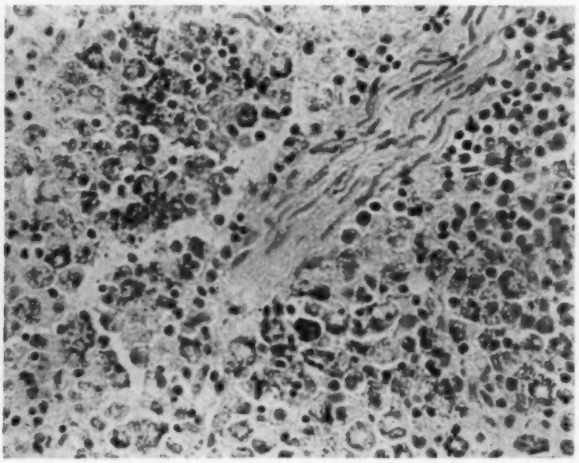

Fig. 8—Rod-containing macrophages in the submucosa of the ileum have extensive infiltration as seen in disruption of the muscularis mucosa (group I). Carbol-fuchsin and hematoxylin; x 330.

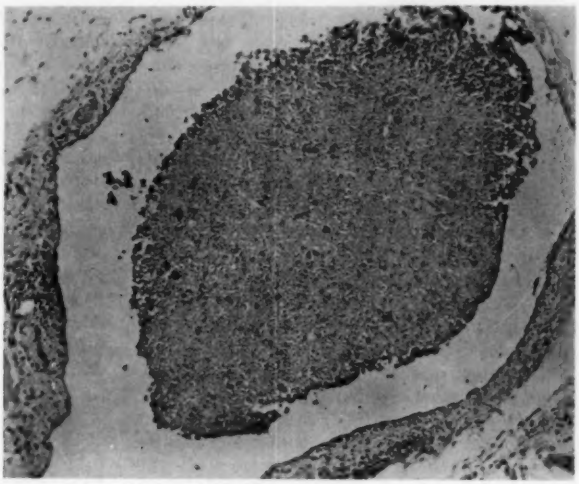

Fig. 9—Thrombus in the vein of serous layer of ileocecal valve (group I). H & E stain; x 90.

disturbed ingestion and absorption, leading to the depletion of plasma protein as pointed out by McFadyean [17] and Runnells.[24]

There appears to be unanimity in the literature insofar as progressive emaciation is concerned. Marked emaciation in sheep of group I and less in those of groups II and III further indicates distinction in the stages of this disease, which might be due to the gradual depletion of fat from fat depots.

The views of previous workers vary with regard to the presence of corrugations in the intestinal mucosa of sheep affected with paratuberculosis. Stockman,[28] Dunkin and Balfour-Jones,[4] Howarth,[11] Nanda and Singh,[20] Stamp and Watt,[27] and Twort and Ingram [30] and Michael [19] reported congestion and hemorrhagic spots of the intestinal mucosa of sheep. Stockman [28] and Dunkin and Balfour-Jones [4] further pointed out absence of corrugations in the mucosae of sheep and considered it to be a feature distinguishing this disease in sheep from that in cattle. The presence of distinctly corrugated mucosae in 3 sheep in the present study does not support the findings of these authors but conforms with the views of Davies,[5] who described the corrugations in the intestinal mucosa.

The different types of other gross lesions in the intestine, such as the thickening and congestion of mucosa observed in this study, are in accordance with the findings of various workers. Most of the investigators have not related their observations to any stage or the progress of the disease, except Michael,[19] who described congested mucous membranes in young sheep during the rapid onset of the disease.

Some authors have regarded the variation in macroscopic lesions to be due to the different strains of *Myco. paratuberculosis*. Sheather [26] stated that there were gross lesions without acid-fast organisms, and also reported no apparent lesions but teeming organisms in the mucosal scraping. He attributed this to variation in the virulence of the strains of organisms or to variation in response to infection. Stamp and Watt [27] concluded that pigmented lesions were due to the pigmented variety of organisms, shown to be more virulent for sheep. They also stated that demonstration of acid-fast bacilli in the terminal part of ileum was not possible in all cases.

Apart from the views of previous workers regarding gross lesions, present observations suggest that the variability of lesions might also be influenced by the stage of the disease. It was observed that sheep in the advanced stage (group I) had highly congested mucosa involving a considerable length of intestine and had large numbers of organisms in smears; in groups II and III, congestion and variable thickening were evident but not so intense, and involved fewer portions of the intestinal wall. The presence of acid-fast bacilli could be demonstrated in only 2 sheep of group II

and in 1 sheep of group III. These facts further indicate that early gross lesions in sheep begin as focal areas of congestion, with or without thickening. The lesions gradually extend, involving larger portions of the small intestines as the disease progresses, until in advanced stages large areas of ileum, jejunum, and duodenum are affected and many organisms are present.

It was found in this study that sheep in the advanced stage (group I) had corrugations of the intestinal mucosa, while others had areas of extreme congestion with variable thickness. There is no satisfactory explanation for such varied lesions in sheep, and further research to elucidate the pathogenesis of such varied lesions is needed. However, the probability of extension and coalescence of congested, thickened lesions forming characteristic corrugations of the mucosa in cattle, as reported by Hallman and Witter,[7] may apply to sheep as well.

Corneal opacity, seen in 1 sheep of this study, was reported by Michael,[19] who described infected sclera and catarrhal conjunctivitis leading to impairment of vision. In the absence of other reports on similar eye lesions in animals with this disease, nothing can be said about the association of these lesions.

The histology of paratuberculosis was considered by Twort and Ingram[30] and McFadyean[18] to be an aggregation of epithelioid cells which appeared in response to *Myco. paratuberculosis* in the intestinal mucosae of cattle. These authors, and Hallman and Witter,[7] considered the absence of necrosis or caseation to be a feature distinguishing paratuberculosis from tuberculosis. Stamp and Watt[27] reported ovine lesions varying from the epithelioid tubercles to the occurrence of caseation, calcification, and even fibrous tissue encapsulation. Runnells[24] regarded the reaction in paratuberculosis as an example of pure, chronic, granulomatous inflammation, characterized by the initial process of reticuloendothelial cell reaction. The present studies also revealed granulomatous inflammation in the intestine, beginning with the initial focus of the macrophages, with or without organisms, and progressing to excessive accumulation of mononuclear phagocytic cells accompanied by desquamation of epithelium, degeneration, and coagulation necrosis with large numbers of acid-fast organisms. Unlike the findings of Stamp and Watt,[27] none of the sheep in the present investigation had caseous or calcified foci in the intestines. The nodular, worm-like lesions, such as those in sheep 315, were so characteristic of "pimply gut" due to Oesophagostomum that they appeared not to have been caused by *Myco. paratuberculosis*.

Increased cellularity in the mucosa of ileum has been considered by most workers as a definite feature of this disease, but there is no unanimity of views regarding the type of cells involved in such a reaction. Hallman and Witter[7] described the progressive increase in the number of macro-

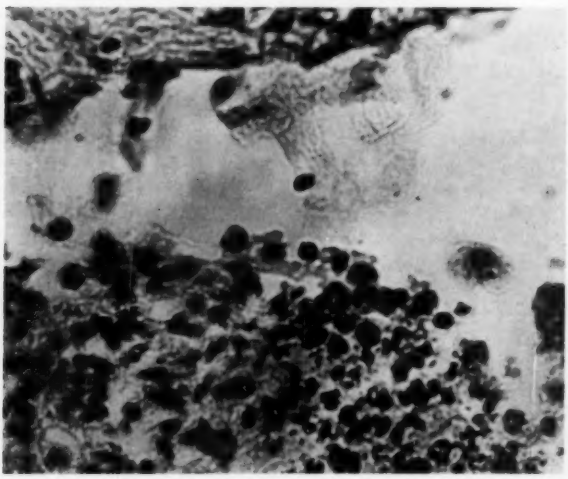

Fig. 10—Higher magnification of figure 11; at the point of attachment of the thrombus are macrophages with intra- and extracellular organisms. Carbol-fuchsin and hematoxylin; x 800.

phages and a variable number of giant cells in cattle. They also observed the presence of neutrophils and believed them indicative of the invasive phase of *Myco. paratuberculosis*. Howarth [11] associated the presence of neutrophils with acid-fast organisms and macrophages in the intestines of sheep. McEwen [15] stated that the lesions of paratuberculosis in sheep, whether in the intestines or lymph nodes, were confined to the accumulation of one type of cell, *i.e.*, the macrophage or large epithelioid cell. He considered that there was no increase of plasma cells, lymphocytes, or eosinophils. Howarth [11] and McEwen [15] further recognized the rare occurrence of giant cells in such lesions of sheep. However, macrophages, epithelioid cells, and giant cells were found to be the chief reacting cells in sheep of group I; in group II sheep, the plasma cells and lymphocytes were predominant, along with some macrophages and a few giant cells; and in group III sheep, lymphocytes were most prominent along with some macrophages. The accumulation of these different types of cells and the absence of neutrophils in sheep in groups I and II conform to the observation of Stamp and Watt,[27] who ascribed the increased cellularity to epithelioid cells, macrophages, giant cells, plasma cells, and lymphocytes.

No plausible explanation has been advanced for the accumulation of these reacting cells, but Hallman and Witter [7] suspected the increased cellularity in cattle to be the result of a transformation of lymphocytes to macrophages and also the multiplication of macrophages themselves. In this study, dividing macrophages with acid-fast rods were also seen in some cases. Forbus [6] explained that a proliferative and phagocytic response in the reticuloendothelial system follows the stimulation of cells of this system by a variety of agents. Such reaction can also be true for paratuberculosis, particularly because this disease is characterized by pure granulomatous inflammation. There occurs an excessive accumulation and proliferation of cells belonging to the so-called "reticuloendothelial system" with a scanty amount of fluid and minimum vascular changes.

From the comparative study, it was clear that the lesions varied from a focal to a diffuse type of reaction. The earliest changes were evident in sheep in group III. The chief cells were macrophages, plasma cells, and lymphocytes in the outer third of the mucosa, without any acid-fast organisms. The cellular increase in most of these sheep caused distention of the villi without desquamation of the epithelium. This observation conforms with that of Hallman and Witter,[7] who described the primary lesions in cattle as foci of one or more macrophages or giant cells with none or a few acid-fast organisms. They also showed that in early lesions of the first stage of the disease there was a small proportion of macrophages and giant cells with acid-fast rods.

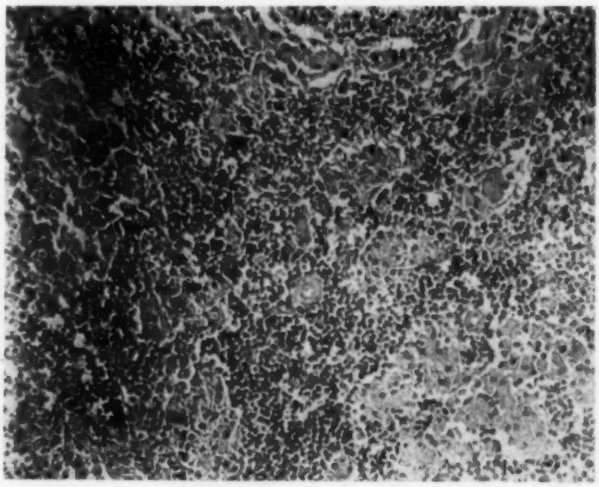

Fig. 11—Ileocecal lymph node; notice diffuse infiltration of macrophages in sinusoids (group I). H & E stain; x 160.

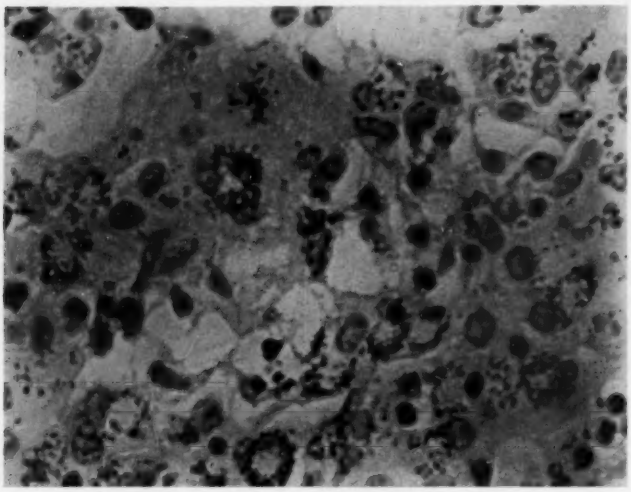

Fig. 12—Acid-fast organisms in macrophages of ileocecal lymph node (group I). Carbol-fuchsin and hematoxylin; x 830.

McEwen [15] described the accumulation of epithelioid cells without any organisms. He suggested that resistance or immunity may sometimes occur and that the bacilli are effectively disposed of, leaving the epithelioid cells as evidence of such activity. On the contrary, Hallman and Witter [7] believed early lesions of the first stage to be due either to stimulation by the organisms, bringing about the increase of macrophages, or the destruction of the engulfed bacteria. Lysis was also pointed out to be apparently lost with the progress of the disease. This seems to be a satisfactory explanation for the fewer numbers of organisms in early lesions. The present study also revealed the possible occurrence of both destruction and stimulation, as evidenced by the presence of acid-fast granules in the cytoplasm of macrophages (sheep 203) and the presence of acid-fast rods in macrophages undergoing division. Stamp and Watt [27] and Harding [8] also recognized acid-fast granules as degenerated or involuted forms of the bacilli.

Although lesions of sheep of the second group were not sharply distinct from those of group III sheep, they served as a cri-

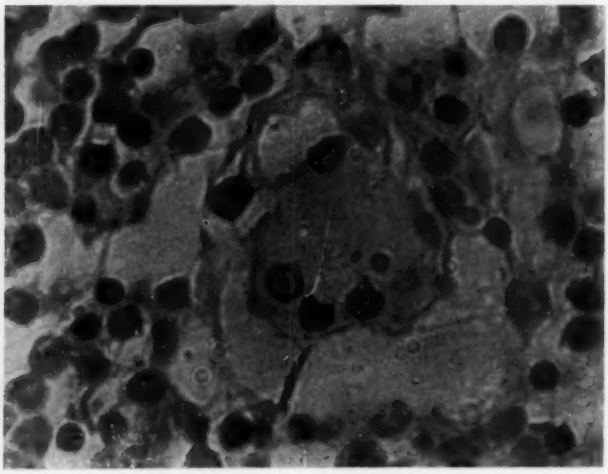

Fig. 13—Giant cell in lymph node (group I). H & E stain.

terion of the progress of reaction, differing in the type of reacting cells, intensity of reaction, effects of reaction, and the number of organisms.

The main reacting cells of group I and II sheep evidently were epithelioid cells, giant cells, and macrophages, with a few lymphocytes and plasma cells. In group III sheep, lymphocytes and plasma cells were the predominating cells with a few macrophages present. The infiltration of the cells extended into the muscularis mucosa and submucosa of the ileum in sheep of groups I and II, being more intense in those of group I than those in group II, with a large number of organisms in sheep in group I. It would appear that the lesions in group I sheep are associated with the advanced stage of the disease. Involvement of the muscularis mucosa and submucosa of the intestines with heavy accumulation of cells has likewise been considered as occurring in advanced stages by Twort and Ingram,[30] Hallman and Witter,[7] and McEwen.[15] In addition to the involvement of mucosa and submucosa, the varied proportion of epithelioid cells, macrophages, plasma cells, and lymphocytes also suggested differentiation of lesions in early and advanced stages of the disease. It appears that in addition to other factors, such as the strain of organism and susceptibility of the host, the stage of the disease might play a role in the variation of lesions so frequently reported in sheep.

The chronic granulomatous type of inflammation of this disease, leading to desquamation of epithelium, symplasm stage, atrophy of crypts, and necrosis of the superficial mucosa (as seen in the group I sheep of the present study), has also been reported by McEwen[15] and Stamp and Watt.[27] The cause of necrosis in the intestinal mucosa appears to be due to pressure atrophy and mechanical blockage of lymph vessels as expressed by McEwen.[15]

Pathologic changes in the lymph nodes in the majority of sheep in this study were enlargement and edema. The involvement of lymph nodes, particularly mesenteric nodes, has been noticed by almost all of the workers. The earlier workers (McFadyean,[16] Beebe,[2] and Twort and Ingram[30]) did not report the caseated and calcified foci in the lymph nodes. McFadyean and Sheather[18] experimentally produced caseous lesions in the lymph nodes of sheep, cattle, and goats. Subsequently, such lesions were reported by Pande[22] and Levi[13] in goats, while Stamp and Watt[27] and Davies[5] mentioned them in sheep. From the observations of these authors and from the present study, it would appear that the presence of caseation and calcification in the lymph nodes of sheep is not uncommon in this disease and these degenerative changes may result from symplasm, casea-

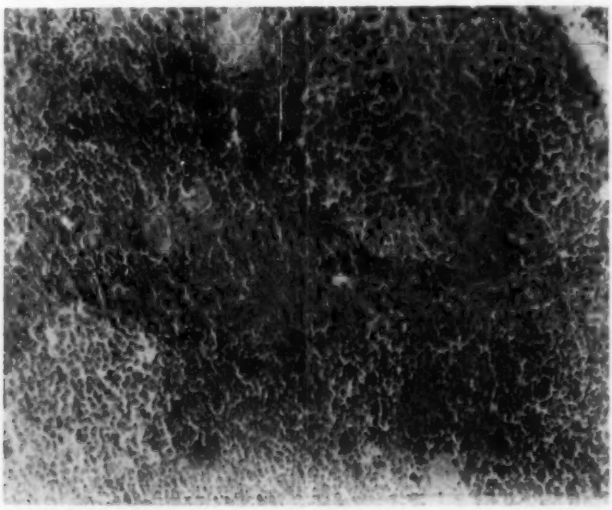

Fig. 14—Accumulation of large mononuclear cells of the germinal center of mesenteric lymph node (group I). H & E stain; x 160.

Fig. 15—Caseous necrosis in mesenteric lymph node (group I). H & E stain; x 160.

tion, calcification, and further encapsulation. Stamp and Watt [27] attributed the possible cause of varied degenerative changes to be due to variations in the strain of the organisms. The secondary effects of profuse infiltration and proliferative reaction of cells, resulting in the nutritional disturbance of both tissues and cells, might also be considered as contributing to necrosis which may be followed by caseation and calcification.

Fig. 16—Mediastinal lymph node showing infiltration of macrophages in the cortex (group I). H & E stain; x 210.

Edema of the lymph nodes has been suggested by Hallman and Witter [7] and Howarth [11] to be due to mechanical disturbances in circulation as a result of accumulation of cells. However, in the present findings, the blood vessels of the lymph nodes were almost normal; therefore, the possibility of obstruction in the flow of lymph as a probable cause of edema may be a better alternative explanation.

Early lesions in the lymph nodes could be seen as aggregations of mononuclear cells in the sheep of group III. In most of the sheep in group I, the changes were diffused in the sinusoids and consisted of macrophages, giant cells, and epithelioid cells, with intracellular acid-fast rods. The normal parenchyma of the lymph nodes was subsequently replaced by the infiltrating cells. These observations conform to the changes described by Hallman and Witter,[7] who also mentioned a diffuse involvement of the nodes by an increased proportion of macrophages and giant cells in relation to the extension of the process. Harding [8] described the symplasm stage of the lymph nodes and considered the medullary changes in lymph nodes in relation to the extensive involvement of the cortex. According to the observations of these authors, the lesions in the animals of group I appear to be advanced and extensive and can well be differentiated from the early lesions of sheep in group III.

Hallman and Witter [7] and Stamp and Watt [27] stated that there seemed to be no positive correlation between the clinical signs, microscopic lesions, and the number of organisms. Harding [8] pointed out a high degree of correlation between the presence of lesions and the isolation of *Myco. paratuberculosis*. The views of Harding are supported by present observations that the presence of organisms varies directly with the pathologic changes and apparent body conditions. These observations are further supported by Twort and Ingram,[30] who stated a positive correlation between the number of organisms and pathologic changes in this disease.

It would appear from the literature that the views of the previous workers are not in complete agreement regarding the routes of spread of infection. Houthis [9] stated that the disease progress is through the

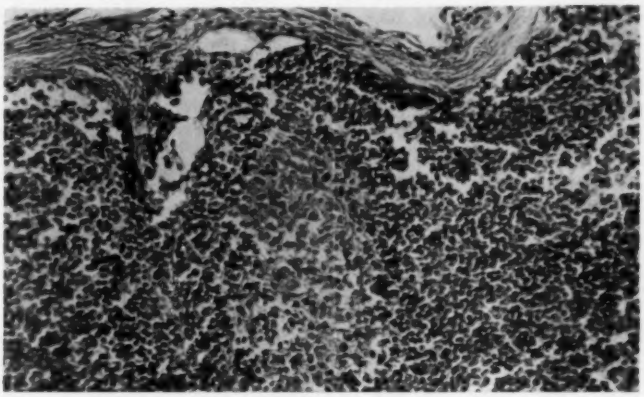

Fig. 17—Early accumulation of macrophages in the cortex of mesenteric lymph node (group II). H & E stain; x 200.

blood vessels in addition to the lymphatic system. Hallman and Witter [7] favored the the lymphogenous route of infection, because they observed the occlusion of lymph vessels. Alexejeff [1] believed the disease to be a bacteremia. In spite of the fact that he reported the isolation of organisms from various organs, milk, and urine, no indication of the progress of organisms in the circulation is obtained from his description. Taylor [29] suggested two possibilities for the spread of the organisms: (1) the organisms are resistant to destruction in the animal body; (2) when in large numbers in mesenteric lymph nodes, they escape into the lymph stream from whence mild bacteremia may arise. Runnells [24] also considered the lymphatic spread in the production of inflammatory reaction in lymph nodes.

Harding,[8] on the basis of experimental studies in goats, stated that the conception of there being only sporadic bacteremic episodes in the advanced clinical stages was wrong and that the occurrence of lesions in organs other than the intestinal tract was hemotogenous and must have occurred early in the disease without the multiplication of organisms. He suggested two obvious routes of dissemination after the ingestion of the bacteria: (1) progressive lymphatic spread, eventually reaching the blood stream through the thoracic duct and hence establishing foci in the lungs, and (2) direct invasion of the portal cir-

Fig. 18—Focal aggregation of macrophages in the ileum (group II). H & E stain; x 200.

culation, indicated by the presence of an infected macrophage in the intrahepatic vessel. The presence of some lesions in the central hepatic vein indicated the probability of the infection gaining access to the systemic circulation through the liver. He pointed out the possibility that extensive lesions in the portal sheath arise by lymphatic spread from intralobular lesions or by systemic spread through the hepatic artery.

This study revealed the obliteration of lymphatic vessels in the submucosa of the intestines; macrophages containing acid-fast bacilli suggested infection of lymph nodes through the afferent lymphatic vessels. The presence of organisms in a thrombus in a vein of the submucosa and also the presence of organisms in the liver and lungs indicate that, in extensive lesions of the advanced stage, the infection might have also extended via blood vessels. Thus, in most cases, the infection spreads through the lymphatics, but when the lesions become extensive, infected macrophages and the organisms may enter the portal circulation and might also be distributed to other organs of the body.

SUMMARY

1) Investigations were carried out on 19 sheep naturally affected with paratuberculosis (Johne's disease). They gave positive reactions to avian tuberculin and johnin. On the basis of clinical signs, the animals were classified as extremely emaciated, slightly weak, and apparently healthy.

2) The pathologic changes in sheep of the 3 groups varied with respect to involvement of the intestines and lymph nodes, the types of reacting cells, intensity of reaction, effects of reaction, and the presence of acid-fast organisms in smears and tissue sections.

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SUMMARIO IN INTERLINGUA

Studios Relative al Pathologia de Morbo de Johne in Oves. III. Alterationes Pathologic in Oves con Infectiones de Occurrentia Natural

1. Esseva effectuate investigationes in 19 oves naturalmente inficite per paratuberculosis (morbo de Johne). Illos manifestava reactiones positive a tuberculina avian e a johnina. Super le base del signos clinic, le animales esseva classificate como extrememente emaciate, levemente debilitate, e apparentemente in bon sanitate.

2. Le alterationes pathologic in oves del tres gruppos variava con respecto al affection del intestinos e del nodos lymphatic, le typos del cellulas reagente, le intensitate del reaction, le effectos del reaction, e le presentia de organismos acido-resistente in frottis e sectiones de tissu.

Bovine Tuberculosis—Studies of Complement-Fixation Antigens

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THE PROGRAM to eradicate bovine tuberculosis in the United States involves the use of intradermal tuberculin tests, the slaughter of all reactor cattle, and the disinfection of the premises. A problem in such an eradication program is that cattle may be tuberculin reactors but have no visible lesions, or they may be nonreactors but have generalized lesions.[9]

Many reports concern the use of various antigen preparations in complement-fixation tests for tuberculosis in man and animals.[3, 5–8, 10, 11, 13, 14] The attempts to develop a suitable complement-fixation test have been only partly successful, mainly because the antigens were either anticomplementary or were lacking in potency or specificity. Serologic tests for bovine tuberculosis have never reached the stage where they can be used routinely in the diagnostic laboratory.

Work with antigens from diseased tissue led Boquet and Negre [1] and Sigurdsson [12] to believe that the complement-fixation value of an antigen depended upon the number of acid-fast bacilli in the diseased tissue. Consequently, in the work reported here, tissue from normal cattle and from cattle that had been sensitized with heat-killed Calmette-Guerin bacillus (BCG) was mixed either with tubercle bacilli or with products of the tubercle bacilli to prepare antigens for the complement-fixation test.

MATERIALS AND METHODS

Diluent.—The diluents were prepared by dissolving 8.5 Gm. of sodium chloride and 0.1 Gm. of magnesium chloride in 1,000 ml. of distilled water.

Antigens.—The 114 antigens were prepared from the following basic materials:

1) *Mycobacterium tuberculosis* var. *hominis, bovis,* and *avium.* Each variety was grown on a synthetic medium, killed with heat, washed in NaCl solution, and dried in an oven at 45 C. It was then ultrasonically treated in saline suspension in a Raytheon oscillator at 10 kc. per second for 1 hour to disrupt the cells.

2) Human tuberculin, bovine tuberculin, avian tuberculin, and a purified protein derivative (PPD) of human tuberculin.

3) Normal bovine tissues, including Bacto liver, dried liver, fresh liver, Bacto-beef heart, dried heart, fresh heart, Bacto-veal, dried muscle, fresh muscle, and dried intestinal mucosa.

4) Dried intestinal mucosa from cattle that had been sensitized with heat-killed BCG in mineral oil.

The antigens were prepared from each basic material and combinations of the materials by Sigurdsson's method.[12] Briefly, the method is as follows: antigen suspension was adjusted to pH 4.3 with HCl, allowed to stand at room temperature for 30 minutes, and centrifuged; the supernatant fluid was discarded. The sediment was resuspended in the same volume of saline solution, adjusted to pH 9.5 with NaOH, refluxed for 1 hour at 100 C., and centrifuged. The sediment was discarded, and the supernatant fluid was adjusted to pH 7.0 with HCl for use as the undiluted antigen.

Serum.—Serum specimens were obtained from cattle in the following 6 groups: (1) animals artificially sensitized either with heat-killed *Myco. tuberculosis* var. *hominis, bovis* (BCG), and *avium,* or with heat-killed *Mycobacterium phlei*; (2) reactors to intradermal tuberculin tests that had lesions at necropsy; (3) normal animals or nonreactors to intradermal johnin and tuberculin tests; (4) reactors to intradermal johnin tests that were microscopically positive for *Mycobacterium paratuberculosis*; (5) reactors to intradermal johnin tests that were microscopically negative for *Myco. paratuberculosis*; and (6) nonreactors to intradermal johnin tests that were microscopically negative for *Myco. paratuberculosis* (animals in groups 5 and 6 were from herds with a history of Johne's disease).

Thimerosal was added as a preservative in the ratio of 1:10,000, and the serums were stored at 4 to 8 C. All serums were diluted 1:5 in saline solution on the day of the test and inactivated at 60 C. for 30 minutes to remove complement and anticomplement. Inactivation at 56 C. for 30 minutes destroyed the complement but not the anticomplement.

Our unpublished studies and the results of other workers [2–4, 12] have shown that diluted bovine serums heated at 60 to 62.5 C. for 30 to 60 minutes were satisfactory for use in the complement-fixation test.

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From the Regional Animal Disease Research Laboratory, Agricultural Research Service, USDA, Auburn, Ala.

The commercial products used in this study were as follows: Raytheon Oscillator Model DF 101, 10 kilocycles, Waltham, Mass.; Bacto preparations, produced by Difco Laboratories, Detroit, Mich.; and Merthiolate (thimerosal), produced by Eli Lilly and Co., Indianapolis, Ind.

TABLE 1—Method of Preparing 13 Antigens Used in Complement-Fixation Tests for Tuberculosis

Antigen No.	Preparation
173	*hominis* PPD (2 mg./ml.), plus 2.0% Bacto-beef heart, refluxed.
175	*hominis* tuberculin (1/4 OT), plus 2.0% Bacto-beef heart, refluxed.
181	*hominis* PPD (2 mg./ml.), plus 2.0% normal dried bovine intestinal mucosa, refluxed.
185	*hominis* tuberculin (1/4 OT), plus 2.0% normal dried bovine intestinal mucosa, refluxed.
179	1.0% *hominis* tubercle bacilli ultrasonically treated, refluxed.
171	1.0% *hominis* tubercle bacilli ultrasonically treated, plus 2.0% Bacto-beef heart, refluxed.
177	1.0% *hominis* tubercle bacilli ultrasonically treated, plus 2.0% normal dried bovine intestinal mucosa, refluxed.
302	1.0% bovine tubercle bacilli ultrasonically treated, refluxed.
304	1.0% bovine tubercle bacilli ultrasonically treated, plus 2.0% Bacto-beef heart, refluxed.
308	1.0% bovine tubercle bacilli ultrasonically treated, plus 2.0% normal dried bovine intestinal mucosa, refluxed.
502	1.0% avian tubercle bacilli ultrasonically treated, refluxed.
504	1.0% avian tubercle bacilli ultrasonically treated, plus 2.0% Bacto-beef heart, refluxed.
508	1.0% avian tubercle bacilli ultrasonically treated, plus 2.0% normal dried bovine intestinal mucosa, refluxed.

PPD = Purified protein derivative; OT = Old tuberculin.

Complement.—Bacto-complement (Difco) was used in all tests. Immediately before each test, a standard complement titration was performed in the presence of an antigen, and the complement was diluted with saline solution so that 0.25 ml. contained 1.5 units.

Erythrocytes.—Sheep blood was obtained aseptically from normal sheep and preserved in Alsever's solution for at least 4 days. The mixture was washed in saline solution 4 times and used in a suspension. This suspension had an optical density of 0.5 ± 0.01 when 0.5 ml. of the erythrocyte suspension was mixed with 5 ml. of distilled water and measured at 580 mμ in a Bausch and Lomb Spectronic 20 colorimeter.

Hemolysin.—Bacto-antisheep hemolysin (Difco) was used in all tests. Immediately before each test, a standard hemolysin titration was performed in the presence of excess complement. The hemolysin was diluted with saline solution so that 0.25 ml. contained 3 units. Sensitization was accomplished by mixing equal parts of the erythrocyte suspension and the hemolysin dilution. This mixture was incubated for 20 minutes at 37 C.

Test Technique.—Five-tenths milliliter of the appropriate serum dilution and 0.25 ml. (2.0 units) of antigen were added to each tube. The tube was shaken immediately and allowed to stand at room temperature for 30 minutes. Then, 0.25 ml. (1.5 units) of complement was added; the tube was shaken and incubated at 37 C. for 30 minutes. After this fixation period, 0.5 ml. of the sensitized cell suspension was added; the tube was shaken and incubated at 37 C. for 30 minutes. The tubes were placed in the refrigerator at 4 to 8 C. and read the next morning after the erythrocytes had settled. The titer of the serum was recorded as the highest dilution without hemolysis. The appropriate controls of the serum, antigen, complement, hemolytic system, and erythrocyte suspension were included in each test. Known positive and known negative serums were used in each test.

For screening, serial twofold dilutions of antigens were tested against a constant dilution of serums from cattle that were sensitized with *Myco. tuberculosis* var. *bovis* and had high, medium, and low complement-fixation titers. Antigens not associated with low titer serums were discarded. Serial twofold dilutions of the best antigens were tested against serial twofold dilutions of known positive and known negative serums. The highest dilution of a given antigen that gave the highest

TABLE 2—Summary of Serial Twofold Dilutions of Antigens when Tested Against Serial Twofold Dilutions of Serums from Tuberculosis-Sensitized Cattle

Antigen source	Antigen No.	Antigen dilution containing 1 unit	Bovine tuberculosis infected cattle			BCG-sensitized cow	Nontuberculous cow	Antigen dilution anticomplementary
			C7	C13	C29	C 1415	C 1607	
Myco. tuberculosis var. *hominis*	173	1:32*	N**	5†	20	40	N	None at 1:4
Myco. tuberculosis var. *hominis*	175	1:64	N	5	20	40	N	None at 1:4
Myco. tuberculosis var. *hominis*	181	1:64	N	5	20	40	N	None at 1:4
Myco. tuberculosis var. *hominis*	185	1:64	N	5	20	40	N	None at 1:4
Myco. tuberculosis var. *hominis*	179	1:64	N	5	40	80	N	None at 1:4
Myco. tuberculosis var. *hominis*	171	1:128	N	10	40	80	N	None at 1:4
Myco. tuberculosis var. *hominis*	177	1:128	N	20	80	160	N	None at 1:4
Myco. tuberculosis var. *bovis*	302	1:32	5	10	80	160	N	None at 1:4
Myco. tuberculosis var. *bovis*	304	1:128	5	20	80	160	N	None at 1:4
Myco. tuberculosis var. *bovis*	308	1:128	5	20	80	160	N	None at 1:4
Myco. tuberculosis var. *avium*	502	1:64	N	10	80	160	N	1:4
Myco. tuberculosis var. *avium*	504	1:64	N	10	40	160	N	1:8
Myco. tuberculosis var. *avium*	508	1:64	N	10	80	160	N	1:8

* Shows highest antigen dilution that gave the highest positive serum dilutions.

** N = Negative, more than 50.0 per cent hemolysis in 1:5 serum dilution.

† Number indicates the highest serum dilution that showed no hemolysis when 1 unit of antigen was used.

serum titer value was considered to be 1 unit. Two units of antigen were used in titrating the serums. The best *Myco. tuberculosis* var. *hominis* and *bovis* antigens were tested against serums from normal cattle, known tuberculous cattle, and cattle sensitized with other acid-fast bacilli. The best antigen was selected on the basis of its potency and specificity.

RESULTS

The methods of preparing the 13 best antigens are shown (table 1). The results from serial twofold dilutions of these antigens when they were tested against serial twofold dilutions of serums from sensitized cattle are also shown (table 2). The best antigens, according to increasing sensitivity, were as follows: (1) ultrasonically treated tubercle bacilli, refluxed; (2) ultrasonically treated tubercle bacilli plus beef heart, refluxed; (3) ultrasonically treated tubercle bacilli plus normal dried intestinal mucosa, refluxed. The antigens from *Myco. tuberculosis* var. *avium* were anticomplementary in 1:4 and 1:8 dilutions. The serum from the BCG-sensitized cow had higher titers for all antigens than did the serums from animals naturally sensitized with tuberculosis.

A comparison of the antigens of *Myco. tuberculosis* var. *hominis* and *bovis* on serums from cattle sensitized to various acid-fast bacilli is shown (table 3). The antigen of *Myco. tuberculosis* var. *hominis* was more sensitive than var. *bovis*, since it gave higher titers in 12 of 47 serums from cattle with tuberculous lesions at necropsy. The latter gave a higher titer in only 1 serum. Both antigens gave complement-fixation

TABLE 3—Comparison of Antigens from Mycobacterium tuberculosis var. hominis (Antigen 177) and bovis (Antigen 308) on Serum from Cattle Sensitized to Various Acid-Fast Bacilli

Classification of cattle	No. of serums	Tuberculosis antigens	
		No. 177 1:64 (2 units)	No. 308 1:64 (2 units)
Intradermal tuberculin-positive			
Tuberculosis lesions, slight	2	N*	N
Tuberculosis lesions, slight	1	N	5**
Tuberculosis lesions, slight	1	S†	S
Tuberculosis lesions, slight	2	5	N
Tuberculosis lesions, slight	2	5	S
Tuberculosis lesions, slight	8	5	5
Tuberculosis lesions, slight	3	10	5
Tuberculosis lesions, slight	2	10	10
Tuberculosis lesions, slight	2	20	20
Tuberculosis lesions, slight	1	80	80
Tuberculosis lesions, marked	2	5	S
Tuberculosis lesions, marked	2	5	5
Tuberculosis lesions, marked	3	10	10
Tuberculosis lesions, marked	3	20	20
Tuberculosis lesions, extensive	1	S	S
Tuberculosis lesions, extensive	1	5	S
Tuberculosis lesions, extensive	3	5	5
Tuberculosis lesions, generalized	1	5	5
Tuberculosis lesions, generalized	1	10	5
Tuberculosis lesions, generalized	1	20	10
Tuberculosis lesions, generalized	3	20	20
Tuberculosis lesions, generalized	2	40	40
Vaccinated, BCG	1	10	20
Vaccinated, BCG	2	40	20
Vaccinated, BCG	1	40	40
Vaccinated, BCG	2	160	160
Vaccinated, *Myco. phlei*	1	20	20
Vaccinated, *Myco. tuberculosis* var. *hominis*	1	20	10
Vaccinated, *Myco. tuberculosis* var. *avium*	1	5	N
Intradermal johnin-positive			
Microscopically positive	1	S	N
Microscopically positive	1	10	10
Microscopically positive	1	20	10
Microscopically positive	3	20	20
Microscopically negative	1	5	5
Microscopically negative	1	40	40
Intradermal johnin-negative			
Microscopically negative	1	20	20
Intradermal tuberculin and johnin-negative	32	N	N

* N = Negative, more than 50 per cent hemolysis in 1:5 serum dilution.
** Number indicates highest serum dilution that showed no hemolysis.
† S = Suspicious, 5.0 to 50.0 per cent hemolysis in 1:5 serum dilution.

titers on serums from cattle sensitized with *Myco. paratuberculosis*. The 6 cattle sensitized with BCG developed higher titers than did cattle naturally infected with tuberculosis.

Discussion

In this study, there was a wide range of sensitivity in the 114 antigens that were tested. The addition of bovine tissues to tubercle bacilli increased the titers of all antigens, and the addition of normal dried bovine intestinal mucosa made the best antigens.

The best antigen was prepared from a mixture of *Myco. tuberculosis* var. *hominis* and normal dried bovine intestinal mucosa and then refluxed. This antigen appeared to be an improvement over other diagnostic or complement-fixation antigens previously reported in the literature [3, 5, 6, 11, 13] for the diagnosis of bovine tuberculosis. In serum from 47 tuberculin reactor cattle with tuberculous lesions, there were 42 positive complement-fixation titers with use of this antigen, and there were no false-positive titers in the serums from 32 normal cattle. The reactors to johnin tests also gave positive complement-fixation titers, which means that bovine tuberculosis cannot be differentiated from paratuberculosis with this antigen. However, preliminary work with a paratuberculosis antigen indicates that this may be accomplished, and further work is being done on this phase.

Summary

In a study of the complement-fixation test for bovine tuberculosis, 114 antigens were prepared from various combinations of bovine tissues, tubercle bacilli, and their metabolic products. The antigens were screened by testing with serums from: (1) cattle naturally infected with bovine tuberculosis; (2) cattle sensitized with heat-killed Calmette-Guerin bacillus; and (3) normal cattle.

The best antigen was contained in the supernatant fluid from a mixture of normal dried bovine intestinal mucosa and *Mycobacterium tuberculosis* var. *hominis* which had been exposed to ultrasonic vibrations. The mixture was refluxed, adjusted for pH, and centrifuged. An antigen of nearly equal activity was prepared in the same manner from *Myco. tuberculosis* var. *bovis*.

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Summario in Interlingua

Tuberculosis Bovin—Studios de Antigenos a Fixation de Complemento

In un studio del test a fixation de complemento pro tuberculosis bovin, 114 antigenos esseva preparate ab varie combinationes de tissus bovin, bacillos tuberculotic, e lor productos metabolic. Le antigenos esseva probate in tests con seros ab (1) bestial naturalmente inficite

de tuberculosis bovin, (2) bestial sensibilisate con thermo-occidite bacillo de Calmette-Guérin, e (3) bestial normal.

Le melior antigeno esseva continite in le fluido supernatante ab un mixtura de normal siccate mucosa intestinal bovin con *Mycobacterium tuberculosis* var. *hominis* que habeva essite exponite a vibration ultrasonic. Le mixtura esseva refluxate, adjustate pro pH, e centrifugate. Un antigeno de quasi equal grados de activitate esseva preparate in le mesme maniera ab *Myco. tuberculosis* var. *bovis*.

The Effects of Exogenous Testosterone on Spermatogenesis of Bulls

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It is generally accepted that, in mammals, spermatogenesis is controlled primarily by 2 pituitary gonadotrophins: follicle stimulating hormone (FSH) and luteinizing hormone (LH), also referred to as interstitial cell stimulating hormone (ICSH). These have been obtained in relatively pure form from pituitaries of swine, horses, sheep, and man.[36] Their use has shown that FSH affects spermatogenesis by direct germinal cell stimulation, and LH affects it indirectly by increasing testosterone production of the steroid-producing testicular interstitial cells, which then enhances spermatogenesis.[23, 34]

Ludwig [25] and Wainman, Reese, and Koneff [37] showed that testosterone maintained spermatogenesis in the hypophysectomized rat but did not initiate spermatogenesis after it had ceased. The pituitary gonadotrophins are necessary, therefore, to initiate spermatogenesis.

Exogenous testosterone affects both steroid-producing testicular interstitial cells and germinal epithelium of the testes of the rat, man, and chicken.[10, 12, 19, 21, 27–29, 37] Large doses of testosterone caused less degeneration of the testes than small doses.[25, 29] Heller *et al.*[14] and Ludwig [25] indicated that injection of testosterone affects the number or function, or both, of steroid-producing testicular interstitial cells. Prolonged testosterone administration inhibits spermatogenesis in man and the rat.[6, 12–14, 22, 23, 37] Permanent tubular degeneration due to testosterone administration has been demonstrated in young male rats but, in mature male rats, the degeneration was temporary followed by a return of tubular function.[12, 14, 27] There is no apparent general agreement on the dosage of testosterone required to depress spermatogenesis in any species, as indicated by the range of dosage used in man.[6, 12, 32]

Injected testosterone apparently inhibits the formation, release, or both, of pituitary gonadotrophins, as evidenced by testicular damage.[6, 9] Injection of appropriate doses of gonadotrophic hormones along with testosterone prevents the testicular damage, thus showing that the damage is not a direct effect of testosterone on the testicular tissue.

Following testosterone withdrawal in man and rats, there is a tendency for a "rebound phenomenon," wherein spermatogenesis increases above preinjection levels.[12–14, 27, 32] There is no similar information available concerning the bull. The "testicular rebound phenomenon" is based on the theory that the sudden cessation of testosterone administration releases pituitary gonadotrophins to stimulate steroid-producing testicular interstitial cells and seminiferous tubules. Work in cases of infertility in man indicated that, although response is not consistent, the improvement of spermatogenesis is sufficiently frequent to warrant testosterone therapy to improve spermatogenesis.[6, 13, 14, 32]

Hooker [16] traced the development of seminiferous tubules and steroid-producing testicular interstitial cells in bulls from birth to 15 years of age. This study indicated that steroid-producing testicular interstitial cells matured in bulls at about 8 months of age, while tubules reached adult "proportions" at about 13 months of age. This work was substantiated by Santamarina and Reece.[31]

Johnsen [18] indicated that it takes about 48 days for the germinal epithelium of a bull testicle to produce spermatozoa; the delay in the recovery of numbers of spermatozoa per ejaculum following withdrawal of testosterone would, therefore, depend on the state of regression of germinal epithelium at withdrawal.

The evaluation of semen by its ability to fertilize ova is the ultimate test. This criterion is too expensive and time-consuming in cattle; therefore, a more practical criterion is necessary. Although no other single criterion of semen evaluation is completely satisfactory, volume, number of spermatozoa per milliliter, total number of spermatozoa per ejaculum, and motility are the most valuable characteristics presently available.[2–4, 11, 22, 35]

This experiment was designed to study the effects of large doses of testosterone for 18 weeks on spermatogenesis and testicular cytology in the bull. Since spermatogenesis in the bull requires at least 7 weeks, ample time for checking the effect was provided. Furthermore, spermatogenesis and testicular cytology were studied by semen evaluation and testicular biopsy after cessation of testosterone injections. Such a study

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TABLE 1—Analysis of Weekly Semen Volume, Spermatozoa per Milliliter, Total Spermatozoa per Ejaculum, Motility Rating, and Abnormal Forms for 4 Hereford Bulls

Measurement	Control (17 weeks) Jan. 10, 1958–May 1, 1958					Testosterone (18 weeks) May 8, 1958–Sept. 5, 1958					Post-treatment (37 weeks) Sept. 12, 1958–May 2, 1959				
	No.	Mean	s.d.	Range	C	No.	Mean	s.d.	Range	C	No.	Mean	s.d.	Range	C
Volume (ml.)	64	4.06	1.70	1.50 to 8.25	42	67	4.43	2.01	1.50 to 8.50	45	142	4.00	2.04	1.00 to 9.50	51
Spermatozoa/ml. (x10⁶)	64	916.10	525.90	40.00 to 2,450.00	57	67	634.60	454.70	40.00 to 2,240.00	72	142	816.55	595.80	10.00 to 2,690.00	73
Total spermatozoa/ ejaculum (x10⁶)	64	3,885.30	3,234.30	140.00 to 1,295.00	83	67	3,240.90	3,242.60	80.00 to 13,760.00	101	142	3,589.93	3,508.60	30.00 to 14,820.00	97
Motility rating	64	2.94	.....	0–4	....	67	2.87	.....	0–4	.....	142	2.65	.....	0–4	....
Abnormal forms (%)	64	17.09	.....	4–60	....	67	18.54	.....	5–45	.....	142	23.75	.....	7–96	....

Measurement	Post-treatment (weeks 1–11) Sept. 12, 1958–Nov. 21, 1958					Post-treatment (weeks 12–37) Nov. 26, 1958–May 23, 1959				
	No.	Mean	s.d.	Range	C	No.	Mean	s.d.	Range	C
Volume (ml.)	41	3.29	2.20	1.00 to 9.50	67	101	4.29	1.91	1.50 to 9.50	45
Spermatozoa/ ml. (x10⁶)	41	355.40	366.50	10.00 to 1,240.00	103	101	1,003.80	569.50	60.00 to 2,690.00	57
Total spermatozoa/ ejaculum (x10⁶)	41	1,139.30	1,331.30	30.00 to 6,110.00	117	101	4,584.80	3,598.90	160.00 to 14,820.00	76
Motility rating	41	2.00	.....	0–4	.....	101	2.92	.....	0–4	....
Abnormal forms (%)	41	32.59	.....	12–96	.....	101	20.16	.....	7–46	....

No. = number of observations; s.d. = standard deviation; C = coefficient of variation.
Unilateral biopsies were performed May 1, 1958, and Sept. 5, 1958.

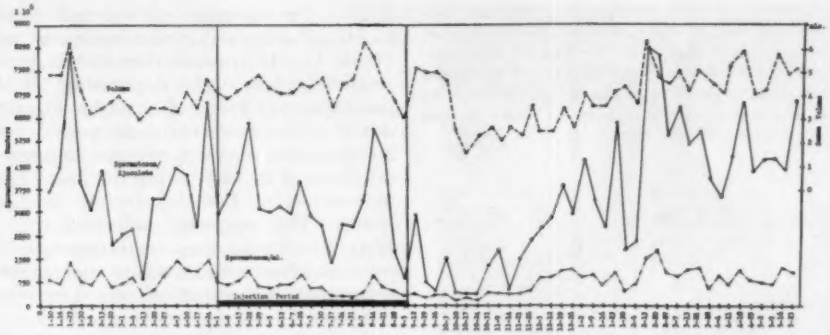

Fig. 1—Weekly average values of semen collected from 4 Hereford bulls (calculated for the number obtained if 4 samples were not collected).

should elucidate some basic endocrine patterns in the bull on which rational experimental and clinical work may be based.

MATERIALS AND METHODS

Seven Hereford range bulls, 21 to 26 months old at the beginning of the experiment, were selected according to physical and genetic uniformity. Since 2 were discarded because of temperament, and 1 due to complete lack of libido, the data presented in this study were obtained from 4 bulls. The body weight of the bulls averaged 1,350 lb. at the end of the experiment.

The bulls were kept in a ½-acre exercise lot with a 16 by 16 ft. enclosed shed provided with an evaporative cooler for year-around protection. The daytime temperature inside the shed did not exceed 85 F.; hence, temperature effects on spermatogenesis were minimized.[5, 7, 29] The bulls were fed a ration of prairie hay and protein-vitamin-mineral supplement at a rate to permit normal growth.

The experiment was begun on January 10, 1958, following an 8-week trial period, during which the bulls became accustomed to surroundings, personnel, and ejaculation into the artificial vagina. The first 17 weeks of the experiment was the control period wherein weekly semen samples were evaluated, 1 unilateral testicular biopsy was obtained from each bull, and 2 control measurements of each testicle were recorded. The treatment period lasted 18 weeks, during which time weekly semen collections, a testicular biopsy at the end of the period, and periodic testicular measurements were made. Three times a week, each bull was given 250 mg. of testosterone propionate in cottonseed oil intramuscularly.[1, 21] The dosage was calculated from that used in man, with consideration given to body weight differences.[6, 13, 30] During the final 37-week post-treatment period, weekly semen samples were evaluated and periodic testicular measurements were taken. The experiment ended with slaughter of the bulls on May 25, 1959, when sections were taken from each testicle.

Semen was obtained from the bulls by use of the artificial vagina and a teaser cow in an outdoor collecting chute. The artificial vagina was of usual design, except that the funnel continued through the artificial vagina to form an additional lining inside the water jacket. The temperature of the water in the artificial vagina was adjusted for each bull within a range of 41 to 44 C. at the time of service. Since these range Hereford bulls were not halter broken, very little teasing was possible. After the bull entered the alley leading to the cow, the person who collected semen stood between the bull and the cow, attempting to hold the bull back at least 2 minutes; however, the bulls usually mounted and ejaculated soon after reaching the cow. One ejaculum from each bull was obtained weekly for evaluation.

Motility, volume, color, density, and libido were recorded as soon as possible after collection of each sample. Density, motility, and libido were rated 0 to 4, the highest number denoting the most favorable quality. These are arbitrary ratings and do not necessarily agree with those of all workers.[4, 22, 30] Volume was recorded to the nearest 0.25 ml. The semen samples were taken to the central laboratory, where spermatozoa counts were made according to the method of Smith and Mayer.[25] A spermatozoan was recorded as abnormal if it had a curled or curved tail; a double, tailless, or pyriform head; or protoplasmic droplets. The number of spermatozoa per ejaculum was calculated as the product of spermatozoa per milliliter times volume. The sample of semen was gently but thoroughly mixed before each procedure to prevent error due to settling.

Testicular measurements to the nearest 1/16 inch were based on an average of at least 2 measurements of the length and diameter, including the skin coverings. The volume of the testicle was calculated by the formula for a prolate spheroid: $4/3\ pi\ ab^2$, where a is the major semiaxis and b the minor semiaxis. This method assumed that any error due to shape was the same for each testicle.

A needle biopsy was performed in the left testicle of each bull at the end of the control period, and in the right testicle at the end of the treatment period according to the method of McDonald and Hudson.[85] Sections taken at slaughter were from the center and periphery of each testicle and from areas of adhesions.

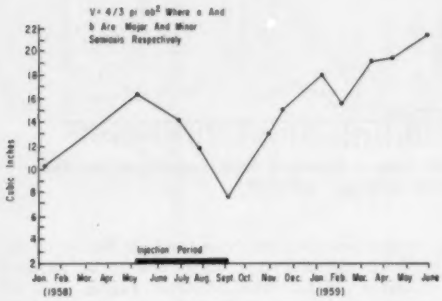
V= 4/3 pi ab² Where a And
b Are Major And Minor
Semiaxis Respectively
Cubic Inches
Injection Period
Jan. Feb. Mar. Apr. May June July Aug. Sept. Oct. Nov. Dec. Jan. Feb. Mar. Apr. May June
(1958)
(1959)

Fig. 2—Average testicular volume for 4 bulls.

RESULTS AND DISCUSSION

The data collected during the experiment were plotted (fig. 1) and are shown (table 1). Examination of the parameters (fig. 1) indicates that semen quality (especially the number of spermatozoa per ejaculum) declined markedly for approximately 11 weeks after discontinuance of the injections, then returned rather quickly to normal or above-normal levels. Therefore, it seemed desirable to separate the data of this 37-week post-treatment period into weeks 1 through 11 and weeks 12 through 37 for further consideration.

The semen volume averaged 4.06 ml. per ejaculum during the control period (17 weeks), 4.43 ml. during the period of testosterone injection (18 weeks), and 4.00 ml. during the post-treatment period (37 weeks). Although no appreciable change in semen volume occurred as a result of experimental procedure, attention should be drawn to the average volume of 3.29 ml. during the first 11-week post-treatment period. Since accessory gland secretion is dependent on androgen, perhaps this post-treatment drop in semen volume occurred because there was a lag period before endogenous androgen production returned following withdrawal of exogenous androgen.

The average number of spermatozoa per milliliter of semen ($\times 10^6$) dropped from 916.10 during the control period to 634.60 during the injection period and returned to 816.55 during the post-treatment period (table 1). It appears that testosterone injection had a slight depressing effect on production of spermatozoa (fig. 1). Breakdown of the post-treatment period reveals an average number of spermatozoa per milliliter of 355.40 during the first 11 weeks in contrast to 1,003.80 during the last 26 weeks. This response indicated that possibly little FSH, LH, or testosterone were present after withdrawal of exogenous testosterone. The question raised is whether temporarily the testosterone injections physiologically hypophysectomized the bulls, insofar as gonadotrophic hormones are concerned, which in turn reduced endogenous testosterone production.

The average number of spermatozoa per ejaculum ($\times 10^6$) was 3,885.30 during the control period, 3,240.90 during the treatment period, 1,139.30 during the first 11 weeks of the post-treatment period, and 4,584.80 in the final 26 weeks. There is little doubt that spermatogenesis was adversely affected by testosterone injections beyond the period of treatment. The rise in spermatozoa per ejaculum following the 11 week post-treatment period (fig. 1) indicated that the depression had been reversed and recovery had occurred. At least a portion of the increased spermatozoa pro-

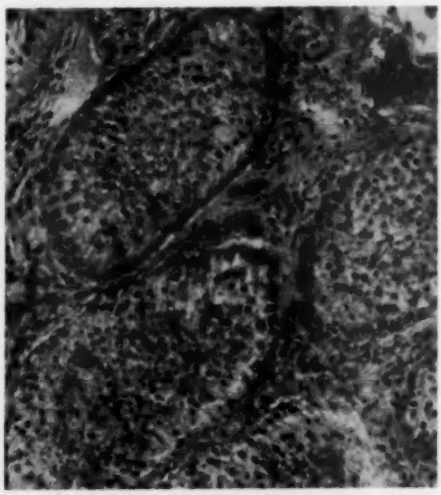

Fig. 3—Section of testicle obtained by needle biopsy before administration of testosterone. H & E stain; orig. mag. x 75.

duction during the post-treatment period must be assigned to the effect of increasing age of the experimental animals.

Motility rating varied from 2.94 during the control period to 2.87 during testosterone injection and 2.65 in the 37-week post-treatment period (2.00 in the first 11 weeks and 2.92 in the final 26 weeks). The motility values declined at the same time the number of spermatozoa per milliliter and per ejaculum declined. The numbers of abnormal spermatozoa increased from 17.09 per cent during the control period to 18.75 per cent during the treatment period (table 1) and 23.75 per cent during the post-treatment period (32.59% during the first 11 weeks and 20.16% during the final 26 weeks of the post-treatment period). Again, some detrimental effects were caused by the high levels of injected testosterone, but more pronounced effects were seen upon withdrawal of treatment and before endogenous production was likely resumed.

The decrease in testicular size during testosterone administration was one of the most profound effects noticed (fig. 2). Spermatogenesis decreased along with the decrease in testicular size, but return of spermatogenesis (fig. 1) followed behind the restoration of testicular volume. Testicular volume increased following withdrawal of testosterone administration, until pretreatment size was reached 3½ months later. Testicular volume returned to near pretreatment levels about December 15, 1958, at which time the semen quality factors also had returned to control levels. Johnsen [18] indicated that at least 48 days are needed for a germinal cell to form a spermatozoan; the remainder of the recovery time may be due to other factors, such as residual testosterone or pituitary recovery.

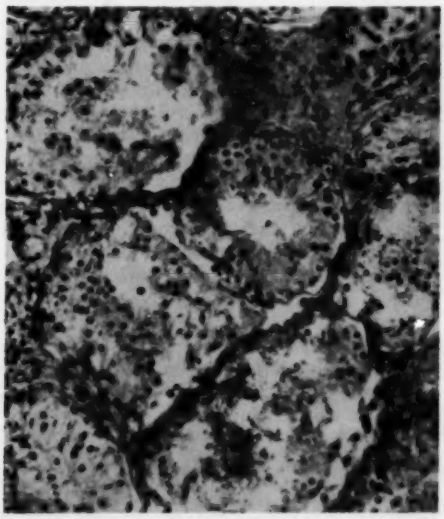

Fig. 4—Section of testicle obtained by needle biopsy following 18 weeks of testosterone administration. Observe the degeneration of the seminiferous epithelium. H & E stain; orig. mag., x 75.

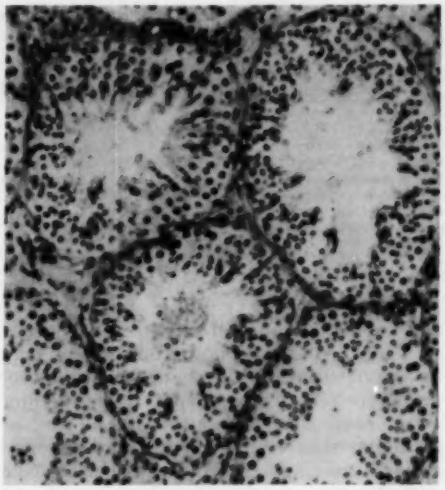

Fig. 5—Section of testicle taken at slaughter (37 weeks post-treatment) showing normal spermatogenesis. H & E stain; orig. mag. x 75.

Biopsy samples at the end of the injection period, Sept. 5, 1958 (fig. 4), revealed marked cytologic changes when compared to the biopsy sections taken at the end of the control period, May 1, 1958 (fig. 3). The epithelium of the seminiferous tubules was so degenerated at the end of the treatment period that fixation and slide preparation were difficult. Some of the separation at the basement membrane must have been an artifact, but arrested spermatogenesis was apparent. Few mature spermatozoa were present. This histologic study was not sufficiently detailed to determine the stage of arrest, but there was indication of detrimental effects on spermiogenesis as well as earlier stages. The decrease in testicular size must have been

TABLE 2—Comparison of Corresponding Seasonal Periods Before and After Testosterone Injections

Measurement	Jan. 10,1958 to May 1, 1958					Jan. 9, 1959 to May 2, 1959				
	No.	Mean	s.d.	Range	C	No.	Mean	s.d.	Range	C
Volume (ml.)	64	4.06	1.70	1.50 to 8.25	42	65	4.60	1.49	2–9.5	32
Spermatozoa/ml. (x10⁶)	64	916.10	525.90	40.00 to 2,450.00	57	65	1,006.46	535.20	110 to 2,690	53
Total spermatozoa/ ejaculum (x10⁶)	64	3,885.30	3,234.30	140.00 to 12,950.00	83	65	4,900.61	2,716.70	220 to 14,060	55
Motility rating	64	2.94	.....	0–4	....	65	3.02	.....	0–4	....
Abnormal forms (%)	64	17.09	.....	4–60	....	65	19.48	.....	7–46	....

No. = number of observations; s.d. = standard deviation; C = coefficient of variation.

a reflection of seminiferous tubule degeneration. Apparently no permanent damage to the spermatogonia occurred, since remarkable recovery occurred in epithelium of the seminiferous tubules (fig. 5) and in testicular size (fig. 2). Study of the biopsy sections taken at the end of the treatment period (fig. 4) revealed less prominent nests of steroid-producing testicular interstitial cells than at the beginning or end of the experiment. The cytologic changes in these cells may have been due indirectly to the exogenous testosterone which blocked pituitary LH production or release, or both, allowing involution of the LH-dependent steroid-producing testicular interstitial cells.

Observation of the bulls' reaction to the teaser cow or other bulls revealed no change in libido during the course of the experiment.

Comparison of Two Corresponding Seasons

Due to the effects of seasonal variations in semen quality,[7,22] 2 equal periods, 1 year apart, were compared in this experiment. The first period was the control period beginning January 10, 1958, and ending May 1, 1958; the second period was during the post-treatment period beginning January 9, 1959, and ending May 2, 1959. It is assumed that weather effects were similar for both periods; the main variable, other than the intervening testosterone injections, was a 1-year age increase.[16,22]

There was an increase from the control period to 1 year later in the average volume of semen from 4.06 ml. to 4.60 ml., in number of spermatozoa (x 10^6) per milliliter from 916.10 to 1,006.46, and in total spermatozoa (x 10^6) per ejaculum from 3,885.30 to 4,900.61 (table 2). Lindley *et al.*[22] and Hooker [16] indicated that such changes could be due to an increase in age of the bulls. The motility value of 2.94 during the control period and 3.02 1 year later, and the percentage of abnormal forms of 17.09 during the control period and 19.48 1 year later (table 2), showed so little change that no importance should be assigned to these subjective criteria of semen quality.

Since the variations in parameters between bulls and within bulls were so large, and since only a small number of animals were used in this experiment, a statistical analysis might not indicate true differences; however, definite biological trends can be observed during the course of the experiment.

Summary

Depression of semen quality factors and testicular size was accomplished in 4 bulls, averaging 1,350 lb. body weight, with 250-mg. doses of testosterone propionate 3 times weekly for 18 weeks. Depression of all values, except testicular volume, continued for approximately 11 weeks after cessation of testosterone injections. Recovery then occurred, until semen quality values were above pretreatment levels although these levels were not statistically significant. The decrease in testicular size was the result of fewer and smaller germinal epithelium cells, as evidenced by biopsy sections. The return of the testicles to normal size began when testosterone injections were discontinued. The testicles regained their pretreatment size before the normal semen qualities were recovered.

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SUMMARIO IN INTERLINGUA

Le Effectos de Exogene Testosterona Super le Spermatogenese de Tauros

Un depression de factores reflectente le qualitate del semine e un reduction del dimensiones testicular esseva effectuate in 4 tauros (de 1.350 lbs de peso medie) per le administration—3 vices per septimana, durante un periodo de 18 septimanas—de doses individual de 250 mg de propionato de testosterona. Le effecto in omne le valores, con le exception del volumine testicular, se manteneva durante approximativemente 11 septimanas post le cessation del injec-

tiones de testosterona. Postea le animales se restabliva usque le valores reflectente le qualitate del semine esseva in supra del valores ante le tractamento, sed iste sur-elevationes non esseva statisticamente significative. Le reduction in le dimensiones testicular esseva le resultato de un reduction in numeros e in dimensiones del cellulas epithelial germinal, secundo le constatationes in sectiones bioptic. Le retorno del testiculos a dimensiones normal comenciava quando le injectiones de testosterona esseva discontinuate. Le testiculos reganiava lor dimensiones pretractamental ante que le qualitates normal del semine esseva restablite.

Chemical and Electrophoretic Studies of the Serums of Cattle Infected Naturally with Asia-1 Type Foot-and-Mouth Disease Virus

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In 1957, Asia-1 type of foot-and-mouth disease (FMD) virus appeared in Israel. At that time, the disease caused by this new type of the virus was limited to cattle of a few villages. In October, 1959, it suddenly reappeared, but took quite a different course, spreading steadily throughout the country and resulting in heavy losses.[3-5] Generally, the clinical signs resembled those induced by other types of FMD virus which did not, however, immunize the animals against the Asia-1 type.

Cattle in 1 of the herds being inspected periodically (in connection with an investigation regarding serum proteins and lipids and their distribution)[8,9] contracted Asia-1 FMD about 1 month after examination. As this presented a unique opportunity, it was decided to study the changes induced by the naturally contracted disease in cattle, the normal blood values of which had been established previously. The examinations were also extended to include the period of recovery.

Materials and Methods

The cattle used in this study were 12 Holstein-Friesian cows, 5 to 9 years old, clinically healthy at the first blood examination, and free from tuberculosis and brucellosis. None had been previously infected with or exposed to FMD virus.

They were examined 1 month before natural infection, 4 to 5 days after the appearance of the first lesions, and again 1 month later. Because 3 of the cows had not recovered clinically (lameness) at the time of the last examination, they were examined 1 month afterward, *i.e.*, 3 months after the first (pre-illness) examination.

Lesions were observed on the oral mucosa inside the lips, on the dorsum of the tongue, on the skin of the udder, and on the feet. The latter produced lameness that was most pronounced in cow 429. Clinical signs were directly related to the lesions and included salivating, eating painfully, refusing food and water, and smacking of lips and tongue. Reduced food consumption resulted in loss of weight. Milk production, too, was adversely affected (fig. 1). The clinical signs disappeared about 10 days after the onset of the first lesions, but unsteadiness and lack of coordination persisted for 2 to 3 weeks in 9 of the cows. In 2 cows (411 and 426), these signs disappeared only after 6 weeks; in cow 429, they persisted even after 3 months. With the exception of cow 429 (fig 1), milk production reverted to pre-disease levels 2 to 3 weeks after the onset of illness.

Protein values were established according to the modified biuret method of Kingsley [6] and Weichselbaum.[13] Protein patterns were studied by paper electrophoretic methods carried out in a Grassman-type chamber. Michaelis buffer of pH 8.6 and ionic strength of 0.1 μ was used. Evaluation was made by direct densitometry of the stained strips followed by planimetration. Total cholesterol was established by the method of Rappaport and Eichhorn,[11] and the paper electrophoretic lipoprotein pattern was determined according to Swahn's method.[12] Analyses were duplicated for each sample.

Results

During the course of the disease, changes occurred in the total serum protein levels as well as in the paper electrophoretic pattern. Nothwithstanding individual differences, the rising or falling trend of the values was established in all cases by comparison with individual values prior to and during the disease. During the disease, a main feature was a decrease in total proteins as well as in albumin; there was an increase in *gamma* globulin (fig. 2–4).

Three of the 12 cows (411, 426, 429) deviated from the main course of recovery (fig. 5–7). Therefore, the results pertaining to 3 cows are presented as individual values while the results from the other 9 are summarized in mean values.

The mean values for total proteins, their albumin/globulin ratio, and their paper-electrophoretic distribution were calculated in percentages of the total (table 1). Also,

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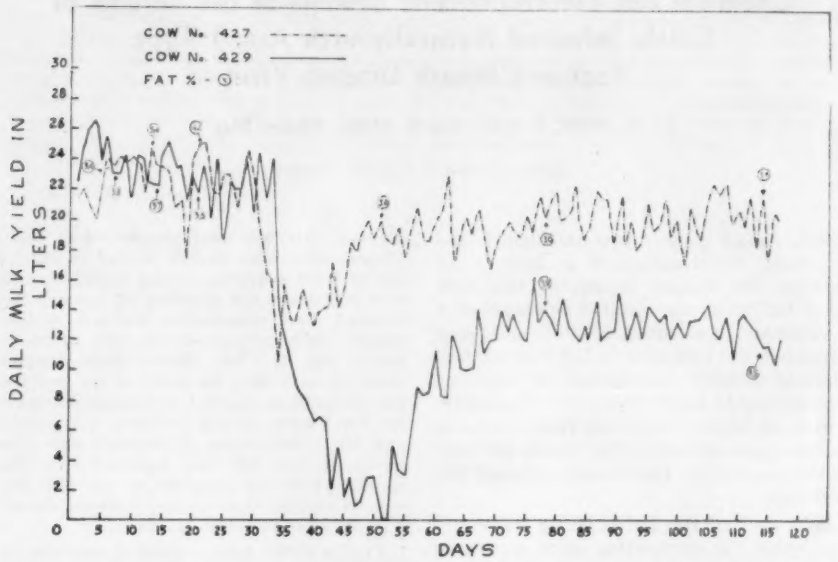

Fig. 1—Daily milk yield of cow 427, representing the main trend (11 cows), and cow 429, the only cow deviating from the main group. Onset of disease was on the 32nd day.

the standard deviation and significance tests were calculated for the 9 cows 1 month before natural infection, 4 to 5 days after the appearance of the first lesion, and 1 month afterward. As seen in table 1, total proteins as well as albumin decreased during the illness. Total protein decreased from 7.56 gr./100 ml. of serum to 6.87 gr./100 ml., and the percentage of the albumin fraction decreased from 45.5 to 38.5. These differences are statistically significant ($P = 0.02$ to 0.05 for total protein and $P = 0.01$ to 0.001 for albumin). At the same time, the relative values of the *gamma* globulin fraction increased significantly ($P = 0.01$ to 0.001) from 28.6 to 35.3 per cent during the disease. The *alpha* globulin increased to a lesser extent (11.3 to 13.6%), while *beta* globulin decreased slightly (from 14.3 to 12.3%). These changes resulted in a decrease of the albumin/globulin ratio during the disease (from 0.83 to 0.62). The differences for *alpha* and *beta* globulins were not statistically significant. At the time of recovery (third blood examination), the mean values for total proteins, as well as their distribution, reverted almost to pre-illness values (in 9 cows). However, the *gamma* globulin, expressed in percentages of the total, was still higher than the pre-illness values but significantly lower ($P = 0.02$ to 0.05) than the corresponding

TABLE 1—Total Serum Proteins and Their Paper-Electrophoretic Distribution (%) of 9 Cows Before Illness, at the Time of Foot-and-Mouth Disease Infection, and One Month Later

Time of examination	Total protein	Albumin	Globulin distribution			Albumin/globulin ratio
			alpha	*beta*	*gamma*	
I. One month before disease	7.56 ± 0.5	45.5 ± 3.7	11.3 ± 0.8	14.3 ± 0.7	28.6 ± 3.3	0.83
II. At onset of disease	6.87 ± 0.6	38.5 ± 4.0	13.6 ± 1.4	12.3 ± 1.8	35.3 ± 4.9	0.62
III. One month later	7.65 ± 0.7	44.7 ± 3.8	12.2 ± 1.0	11.9 ± 2.0	31.0 ± 2.7	0.79
SIGNIFICANCE OF THE DIFFERENCES (MEAN VALUES)						
Between I and II	P = 0.02–0.05	0.01–0.001	.....	.....	0.01–0.001	.....
Between II and III	P = 0.02–0.05	0.01–0.001	.....	.....	0.02–0.05	.....

TABLE 2—Total Serum Proteins and Their Paper-Electrophoretic Distribution in Absolute Values (Grains per 100 ml. of Serum)

Time of examination	Total protein	Albumin	Globulin		
			alpha	*beta*	*gamma*
I. One month before disease	7.56 ± 0.5	3.44 ± 0.28	0.85 ± 0.06	1.08 ± 0.053	2.16 ± 0.25
II. At time of disease	6.87 ± 0.6	2.64 ± 0.27	0.93 ± 0.096	0.84 ± 0.12	2.43 ± 0.34
III. One month later	7.65 ± 0.7	3.42 ± 0.29	0.93 ± 0.08	0.91 ± 0.15	2.37 ± 0.21
SIGNIFICANCE OF THE DIFFERENCES (MEAN VALUES)					
Between I and II	.....	P = 0.001	NS	NS	0.05
Between II and III	.....	P = 0.001	NS	NS	NS

NS = not significant.

levels during the disease. No clinical signs were observed at this time in these cows.

Absolute values, in grains per 100 ml. of serum (table 2), confirmed the trend evidenced in the relative values. The albumin decreased significantly (from 3.44 gr./100 ml. to 2.64 gr./100 ml. during the illness). The increase in *gamma* globulin was less pronounced.

As already mentioned, 3 cows (411, 426, 429) still had clinical signs such as lack of equilibrium and lameness at the third examination. These were especially noticeable in cow 429. Individual blood values for the 3 cows, including values obtained at a fourth examination 1 months after the third, are shown (table 3). One month after the onset of disease, total protein closely approximated pre-illness levels, while the paper-electrophoretic pattern resembled that during the disease.

At the fourth serum examination, values for cows 411 and 426 were found to have reverted almost to pre-illness levels. Also, no clinical signs could be found at this time, and milk production was as high as before illness. At this examination, the serum proteins of cow 429 had not yet reverted to pre-disease levels. Lameness and secondary necrotic lesions on the feet near the hoofs persisted, and milk production was still impaired (fig. 1).

Cholesterol and Lipoprotein Distribution.—In a previous study,[9] the 3 lipoprotein components found were referred to as *alpha, beta,* and *gamma* lipoproteins. In this study, however, during the disease the lipoprotein previously referred to as *gamma* could not be separated from the *beta* lipoproteins (despite efforts with different buffers, running times, and voltages). They were therefore measured together, as is often done in human examinations.[13, 17, 18] These 2 compounds will be referred to as lipoprotein component I (*alpha* lipoprotein) and component II (*beta* and *gamma* lipoprotein).

Mean values for total cholesterol, lipoprotein distribution, and statistical analysis with data from 9 cows is shown (table 4).

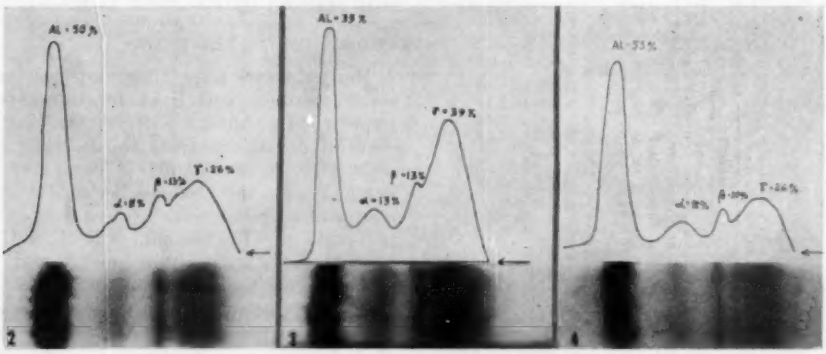

Fig. 2, 3, 4—Paper-electrophoretic patterns (%) obtained from cow 427 (representative of the 9 cows with normal recovery) 1 month before foot-and-mouth disease (2), at the time of illness (3), and 1 month later (4).

TABLE 3—Results of 3 Individual Cases

Cow No.	One month before illness					At time of illness					One month later					Two months later				
			Globulin					Globulin					Globulin					Globulin		
	T.P.	Al.	a	b	g	T.P.	Al.	a	b	g	T.P.	Al.	a	b	g	T.P.	Al.	a	b	g
TOTAL PROTEINS AND THEIR PAPER-ELECTROPHORETIC DISTRIBUTION (%)																				
411	7.3	46.0	10.0	14.0	30.0	6.9	39.5	13.0	13.0	34.5	7.7	39.5	11.5	11.5	37.5	7.3	47.5	11.0	13.0	28.5
426	7.7	44.0	10.5	13.5	32.0	7.2	38.0	15.0	11.0	36.0	7.8	34.5	14.5	15.5	35.5	7.6	38.5	12.5	15.5	33.5
429	8.8	43.0	11.0	13.0	33.0	8.3	30.0	13.5	9.0	47.5	8.8	36.5	9.5	14.5	39.5	8.7	34.0	13.0	13.0	40.0
IN ABSOLUTE VALUES (GRAINS PER 100 ML. OF SERUM)																				
411	7.3	3.3	0.73	1.02	2.2	6.9	2.7	0.9	0.9	2.4	7.7	3.0	0.9	0.9	2.9	7.3	3.5	0.8	0.9	2.1
426	7.7	3.4	0.8	1.04	2.5	7.2	2.7	1.1	0.7	2.6	7.8	2.7	1.1	1.2	2.8	7.6	2.9	0.9	1.2	2.5
429	8.8	3.8	0.9	1.10	2.9	8.3	2.5	1.1	0.7	3.9	8.8	3.2	0.8	1.3	3.5	8.7	3.0	1.1	1.1	3.5

T.P. = total protein; Al. = albumin; a = *alpha*; b = *beta*; g = *gamma*.

The values for cows 411, 426, and 429 are listed separately (table 5). In this instance, in addition to the 3 series of examinations (before, during, and after the disease) which were made for proteins, a fourth test was run 2 months after the onset of the disease. This was of interest because the total cholesterol levels did not revert to pre-illness values after the 1-month recovery period (third examination). At the time of the disease (table 4), total serum cholesterol and *alpha* lipoprotein (component I) decreased from 158.2 mg./100 ml. of serum (pre-illness) to 98.4 mg. at the time of the disease; and for *alpha* lipoprotein from 68.8 to 44.3 per cent. Statistically, these changes were highly significant ($P = 0.001$). Subsequently, the component II lipoprotein increased during the disease from 31.2 (pre-disease) to 55.7 per cent. As a result, the II/I component ratio (quotient) increased from 0.45 to 1.26.

One month after the second examination, the paper electrophoretic lipoprotein components reverted to close to normal levels. Total cholesterol values remained still lower (134.1 mg./100 ml. of serum) than before the disease (158.2 mg./100 ml. of serum). However, there is a statistically significant difference ($P = 0.01$ to 0.001) between the cholesterol values obtained during the disease and those obtained 1 month later.

The fourth examination (2 months after the onset of the disease) did not reveal any changes from the results of the third examination (1 month after the onset of the disease). In 3 cows (411, 426, 429) in which there was deviation in the protein pattern, there was no deviation from the main trend with respect to lipid levels (table 5).

DISCUSSION

Many factors may affect normal bovine serum protein and lipid levels and their paper-electrophoretic patterns, causing substantial deviations from the normal. Consequently, it is difficult to compare, in a general way, the changes occurring in the course of a disease with respect to the "normal values." Factors like age, breed, sex, physiologic status, environment, and food affect the electrophoretic pattern.[2, 7-10, 16]

In the present report, these difficulties were almost absent. In the cows studied, the disease was contracted naturally 1 month after a routine blood examination. The examinations referred to concerned serum proteins and cholesterol and their

paper-electrophoretic distribution. Thus, it was possible to detect even slight changes.

The outstanding fact which became evident immediately is that cows naturally contracting the disease had a highly significant decrease in the albumin fraction, while the globulins, especially *gamma* globulin, increased.

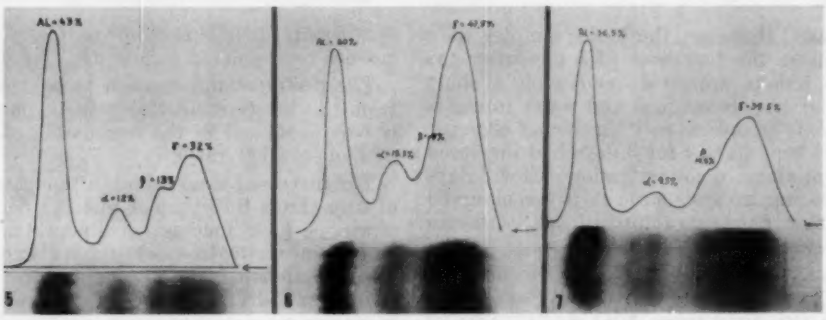

Fig. 5, 6, 7—Paper-electrophoretic patterns (%) obtained from cow 429 (representative of the 3 cows with delayed recovery) 1 month before foot-and-mouth disease (5), at the time of illness (6), and 1 month later (7).

If the absolute values (in gr./100 ml. of serum) and the relative values obtained by paper electrophoresis are compared, it becomes evident that the albumin decrease is to be regarded as the main factor in the decrease of the total protein. The decrease in albumin is generally explained by the need to maintain the colloid osmotic pressure [7, 17, 18] or by liver alternation.

increases during FMD because of the formation of antibodies, especially the *gamma* globulin, the animal organism prevents a simultaneous increase in the colloid-osmotic pressure by a corresponding decrease of the albumin level. Regulation by a change in the albumin level is most effective because albumin exerts the largest colloid-osmotic pressure; it seems that the organism either modifies the albumin genesis or causes its increased storage in the liver. However, the albumin decrease is too great compared with the increase of the *gamma* globulin. Some of these changes are more likely to be due to the fact that the disease, causing metabolic stress, impairs the liver function, since the liver is the site of the synthesis

TABLE 4—Total Serum Cholesterol and Lipoprotein Distribution in Percentage, Standard Deviation and Significance Test

	Total cholesterol (mg./100 ml. serum)	Lipoprotein distribution (%)		
		Component I	Component II	II/I
I. One month before illness	158.2 ± 16.8	68.8 ± 6.2	31.2	0.45
II. At time of illness	98.4 ± 22.9	44.3 ± 10.4	55.7	1.26
III. One month later	134.1 ± 23.6	66.2 ± 8.4	33.8	0.51
IV. Two months later	136.2 ± 19.2	66.3 ± 7.9	33.6	0.51
SIGNIFICANCE OF THE DIFFERENCES (MEAN VALUES)				
Between I and II	P = 0.001	P = 0.001	P = 0.001	.....
Between II and III	P = 0.01–0.001	P = 0.001	P = 0.001	.....
Between II and IV	P = 0.01–0.001	P = 0.001	P = 0.001	.....

The colloid-osmotic pressure of blood plasma represents one of the most important physiologic functions. Therefore, it must be assumed that the organism has a regulative mechanism which assures its constancy.[17] As the globulin in the serum of albumin.[7, 17] Changes in liver function may cause a percentage reduction of the serum albumin concentration.

Another possible cause for the albumin/globulin changes might be the reduced food and water intake during the course of the

TABLE 5—Individual Values for Total Cholesterol and Lipoprotein Distribution

Cow No.	One month before illness			At time of illness			One month later			Two months later		
	Total cholesterol	Lipoprotein			Lipoprotein			Lipoprotein			Lipoprotein	
		I	II	T.Ch.	I	II	T.Ch.	I	II	T.Ch.	I	II
411	128	65.0	35.0	90	23	77	92	50	50	99	49	51
426	191	66.5	33.5	154	47	53	183	62	38	162	78	22
429	154	72.0	28.0	96	43	57	137	56	44	138	67	33

Total cholesterol values are given as milligrams per 100 ml. of serum; lipoprotein components I and II are given in percentages.

disease. However, the blood samples were taken on the fourth or fifth day after the first lesions appeared, and such a short period of reduced food and water intake is unlikely to induce such significant changes. Cows kept thirsty for 3 days had increased serum albumin concentration; after 2 days of fasting, no systematic shift was observed in the concentration of the plasma proteins.[14]

Brandish *et al.*[1] studied the serum protein pattern of steers artificially inoculated with FMD virus types A, O, and C. During the first week of illness in cattle, Brandish *et al.* found a decrease in *gamma* globulin with very little or no change in the albumin. This contradicts the findings of the present authors, who found that during the same period the *gamma* globulin increased and the albumin fraction decreased significantly. However, Brandish *et al.* found that 35 days after inoculation (corresponding to the third blood examination in the present study) an elevation of the *gamma* globulin occurred which was even more pronounced than that of the present study.

These differences may be due to the different responses of animals having contracted FMD naturally and those inoculated with the virus. Other factors responsible for these variations may be the presence of different viruses and the fact that incubation time of the Asia-1 type of the virus seems to be much longer.

The fact that the period of recovery for 3 cows differed from the major course (9 cows) is probably due to secondary bacterial infections (particularly in cow 429).

The majority of reports on human serum lipoprotein distribution concern problems of atheromatosis and liver diseases. Other human diseases have been given less attention, although data are available on diabetes, lipoid nephrosis, myxedema, xanthomatosis, and several other diseases.[13, 17, 18] However, abnormal paper-electrophoretic lipoprotein distribution of bovine serum has not been studied sufficiently.

The pertinent information to be derived from this study of the lipoprotein pattern is best presented in the summation of the component II/I ratio.

The increased ratio found in the instance of FMD (from 0.45 normal to 1.26) demonstrates a high increase of lipoprotein in component II (*beta* and *gamma* lipoprotein fractions). These changes are also found generally in diseases of the liver and in numerous cases where atheromatosis has been clinically proven.

The decrease in albumin protein and the increase of component II (lipoprotein) supports the assumption that alterations occurred in the liver as a result of FMD.

SUMMARY

Chemical and paper-electrophoretic studies of serum obtained from 12 cows that contracted foot-and-mouth disease (Asia-1 type) revealed a significant decrease in total proteins, albumin, and total cholesterol.

Gamma globulin and component II (*beta* and *gamma* lipoproteins) increased significantly during the disease. In 9 of 12 cows, blood values (with the exception of total cholesterol) approximated pre-illness levels 1 month after the onset of illness.

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SUMMARIO IN INTERLINGUA

Studios Chimic e Electrophoretic in Seros ab Bestial Naturalmente Inficite con Virus de Febre Aphthose Typo Asia-1

Studios chimic e studios de electrophorese a papiro in seros obtenite ab 12 vaccas que habeva contrahite febre aphthose (typo Asia-1) revelava un significative reduction in proteinas total, albumina, e cholesterol total.

Globulina gamma e componente II (lipoproteinas beta e gamma) se augmentava significativemente durante le morbo. In 9 de 12 vaccas, le valores hematologic (con le exception de cholesterol total) esseva proxime al nivellos de ante le morbo al fin de 1 mense post le declaration del morbo.

Further Studies on the Survival of Foot-and-Mouth Disease Virus in Meat

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STUDIES on the survival of foot-and-mouth disease virus (FMDV) in cured and uncured meat and a review of the literature were reported recently by Cottral *et al.*[1] These studies showed that FMDV survived for as long as 50 days in lymph nodes packed in barrels with cured and uncured meat. The donor cattle infected with foot-and-mouth disease (FMD) were slaughtered 30 to 35 hours after inoculation with the virus. It was also reported that the virus was present in infected carcasses for as long as 60 days in lymph nodes, blood clots, and muscle samples, and 73 days in bone marrow.

This present report supplements the information on virus survival in lymph nodes under different conditions. The presence of the virus in hemal nodes and bone marrow and the transmission of the disease to swine by feeding bovine bone marrow is also reported.

MATERIALS AND METHODS

The virus used was FMDV, Vallée A type, strain 119, in pooled bovine tongue epithelium from the eighth Plum Island serial passage. The methods for preparation of stock virus and test tissues were similar to those previously reported.[1] The donor cattle were grade Hereford steers, 18 to 20 months old, from the same source as reported previously.[1] The experimental swine were grade Tamworths approximately 12 weeks old.

The right prescapular lymph node was surgically removed, under general anaesthesia, from a steer inoculated with FMDV 20 hours previously. This lymph node was prepared as a sample for subsequent inoculation into 2 test animals.

Lymph nodes, muscle, and bone marrow were removed from another FMDV-infected steer at slaughter, 9 days postinoculation. The prescapular, prefemoral, and popliteal lymph nodes were pooled for this test. Samples of the supraspinatus, the biceps femoris, and the semitendinosus muscles were selected on the basis of their physical relationship to the lymph nodes used. The bone marrow was obtained from the ribs.

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The hemal nodes were removed from a slaughtered steer having signs of FMD and prepared for inoculation into test animals. The fresh hemal nodes were taken from half of the carcass at slaughter and the ripened nodes were taken after the carcass had hung in the cooler for 72 hours at 4 C.

Samples of bone marrow and lymph nodes from an infected steer carcass were tested for FMDV after storage for 194 days at 1 C. The bone marrow was removed from the ribs and vertebras of the ripened carcass; the lymph nodes were taken from the barrel where they were packed with muscle tissue that had been cured with salt for the storage term.

The carcass of an infected steer was allowed to ripen in a cooler for 120 hours. The ribs and spinous process of the thoracic vertebras were then used as a source of bone and marrow for swine feeding. The marrow was scraped from half of the bones, ground in a mortar, suspended in 500 ml. of tryptose phosphate broth and centrifuged at 220 x g for 5 minutes. The supernatant fluid was pooled, and a sample was titrated by inoculation into normal bovine tongues. The other half of the bones containing marrow were split into fragments about 1 inch long and fed to 5 pigs, 12 weeks old, which consumed about 5 lb. of this mixture in 8 hours. As this material was scattered on the floor of the pen, the animals' feet as well as their mouths were exposed to the sharp bony fragments. The bone marrow suspension without the bone fragments was individually fed to a second lot of 5 pigs so that each pig consumed 75 ml.

RESULTS

Infectivity Tests in Steers.—The virus of FMD was demonstrated in the prescapular lymph node surgically removed from a steer 20 hours postinoculation (table 1). This steer was apparently normal and had a normal body temperature at the time the node was removed; no sign of FMD involvement appeared until 4 hours after the operation.

The steer slaughtered 9 days postinoculation no longer had outward signs of FMD, and the body temperature had been normal for 5 days. Virus was demonstrated in the lymph nodes from this animal but not in the fresh muscle tissue and bone marrow samples taken at the same time.

TABLE 1—Survival of Foot-and-Mouth Disease Virus in Tissue from Donor Steers in Various Stages of Infection and Results of Feeding Infected Bone Marrow to Swine

Sample source and treatment	Storage postinoculation	Harvest postinoculation	Infectivity
PRECLINICAL STAGE			STEERS
Lymph nodes, fresh	.....	20 hours	2/2*
CONVALESCENT STAGE			
Lymph nodes, fresh	.....	9 days	2/2
Muscle tissue, fresh	.....	9 days	0/2
Bone marrow, fresh	.....	9 days	0/1
CLINICAL STAGE			
Hemal nodes, fresh	.....	46 hours	2/2
Hemal nodes, ripened**	72 hours	46 hours	2/2
Bone marrow, ripened	194 days	34 hours	2/2
Lymph nodes, ripened	194 days	33 hours	0/2
RIPENED BONES FOR SWINE FEEDING			
Bone marrow control	120 hours	46 hours	2/2
Bone marrow with bone fragments	120 hours	46 hours	SWINE 5/5
Bone marrow, without bone	120 hours	46 hours	0/5

* Numerator = number of animals positive; denominator = number of animals used. ** Ripened indicates that tissues remained in carcass stored at 4 C. for time specified.

Virus was present in the hemal nodes removed from an infected carcass after 72 hours ripening as well as those removed fresh at the time the animal was killed.

Virus was demonstrated in the bone marrow after 194 days storage at 1 C.

The virus was not detected in the lymph nodes stored in the barrel of cured meat for 194 days.

Feeding Trials in Swine.—The marrow supernatant fluid from the carcass ripened 120 hours had a titer of $10^{4.5}$ bovine i.d.$_{50}$ per milliliter. The pigs fed this marrow along with the bone fragments developed FMD within 5 to 6 days. The pigs fed this same material without the bone fragments did not have signs of infection during 15 days of observation. The pigs were susceptible to challenge with strain A-119, using the coronary band titration technique described by Graves and Cunliffe.[2]

DISCUSSION

In studies previously reported by Cottral *et al.*,[1] the infected donor cattle were slaughtered 30 to 35 hours after inoculation with FMDV. During this period, viremia was well established and tests showed the presence of virus in various tissues. Therefore, to determine if the virus would be present in lymph nodes before and after this particular period of viremia, a lymph node was surgically removed from 1 steer 20 hours after inoculation, and several lymph nodes were removed from another steer slaughtered 9 days after inoculation. Virus was found, although the animals in both studies had no signs of infection at slaughter. These results indicate that meat from animals in the stages just preceding and shortly after the regression of signs of FMD would be hazardous to export from countries where FMD is present.

Hemal nodes, as well as the lymph nodes, provide a favorable site for virus survival; the virus was found in both fresh and ripened hemal nodes. These nodes are small and widely distributed throughout the carcass. They are even more difficult to eliminate from meat during trimming than lymph nodes.

The bone marrow apparently offers excellent protection for the virus in FMDV-infected carcasses. Ribs from an infected carcass used in the previous experiments,[1] were stored at 1 C. for 194 days. The virus was found in the marrow of these bones.

Another test used lymph nodes stored for 194 days at 1 C. in a barrel of cured meat from the former experiments. The virus was not found. The virus had been demonstrated in other lymph nodes taken from this same barrel after 30 days' storage and also from another barrel after 50 days' storage.[1]

In a report from Great Britain, Stockman *et al.*[3] observed that the bone marrow was not infective for swine unless bone fragments were included. This observation was confirmed in the studies reported here, using strain A-119. However, this may not be the case for other FMDV strains, particularly those adapted to swine or primarily porcine strains. Furthermore, garbage-fed swine would usually encounter bones or other foreign material sufficient to produce mouth or foot injuries. Therefore, the danger of feeding material contaminated with FMDV to swine should not be minimized.

SUMMARY

Further studies were made on the survival of foot-and-mouth disease virus in fresh, ripened, and stored meat from steers in various stages of infection. New studies were made on transmitting the disease to swine via feed.

Infective virus was demonstrated in (1) a prescapular lymph node which had been

removed from a steer by surgery before clinical signs of disease appeared; (2) lymph nodes, but not in muscle samples or bone marrow, from an animal that had been slaughtered 5 days after regression of disease signs; (3) hemal nodes from both freshly killed and ripened carcasses; (4) bone marrow from ribs and vertebrae of an infected carcass that had been stored 194 days at 1 C., but not in lymph nodes stored in contact with ripened and salted meat in wooden barrels under the same conditions.

Swine developed foot-and-mouth disease when fed infected bone marrow mixed with bone fragments but not when the same bone marrow was fed alone.

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SUMMARIO IN INTERLINGUA

Studios Additional in Re le Superviventia de Virus de Febre Aphthose in Carne de Consumo

Studios additional esseva executate relative al superviventia de virus de febre aphthose in carne de consumo fresc, maturate, e magasinate ab tauros in varie stadios de infection. Nove studios esseva facite con respecto al transmission del morbo a porcos via lor nutrimento.

Virus infective esseva demonstrate (1) in un nodo lymphatic prescapular que habeva essite excidite chirurgicamente ab un tauro ante le apparition de ulle signo clinic del morbo, (2) in nodos lymphatic, sed non in specimens de musculo o medulla ossee, de un animal que esseva abattite 5 dies post le regression de signos del morbo, (3) in nodos hemal tanto de novemente abattite como etiam de maturate cadaveres, e (4) in medulla ossee ab costas e vertebras de un cadavere magasinate durante 194 dies a 1 C, sed non in nodos lymphatic magasinate in contacto con maturate e salinate carne in barriles de ligno sub le mesme conditiones.

Porcos disveloppava febre aphthose quando illos esseva alimentate con inficite medulla ossee in mixturas con fragmentos de osso sed non quando le mesme medulla ossee esseva administrate sol.

Pathology of Epizootic Hemorrhagic Disease of Deer

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THE OCCURRENCE in deer of an epizootic hemorrhagic disease (EHD) of specific viral etiology was first described in 1955 by Shope.[8] He reported on clinical and pathologic findings in white-tailed deer *(Odocoileus virginianus)* involved in an epizootic in New Jersey and described the culture methods used to obtain and characterize the virus.

The same year, Fay and his associates[2] isolated a viral agent from white-tailed deer involved in a similar epizootic in Michigan. A year later, Shope, in cooperation with Berner,[1] isolated a virus from deer dying of a hemorrhagic disease in South Dakota. Recently, Shope[7] has demonstrated distinct serologic differences between the New Jersey and South Dakota isolates of EHD, although the clinical and pathologic characteristics of infections produced by the two agents appear to be similar.

The present study was undertaken to gain more information about the pathogenesis of the disease, especially the histologic changes and the means by which these changes arise.

MATERIALS AND METHODS

The viral isolates used in these experiments were from three sources: the Michigan strain of EHD, obtained from liver tissue of an experimentally infected deer, and the New Jersey and South Dakota serotypes, obtained from the spleens of experimentally infected deer. Serum samples (New Jersey and South Dakota serotypes) were obtained from deer which survived experimental infections. With these, by neutralization in susceptible deer, it was possible to identify the Michigan strain of EHD virus as identical or closely related to the South Dakota serotype.

The 6 deer used in these experiments were 8- to 12-month-old male fawns *(Odocoileus virginianus)* provided by the Wisconsin Conservation Department. All were apparently healthy before experimental infection.

The animals were infected by intramuscular injection of 1 to 10 ml. of 10 per cent suspensions of infective spleen in nutrient broth. To avoid the possible growth of bacterial contaminants, 5 mg. of streptomycin and 5,000 units of penicillin were added per milliliter of inoculum. Following exposure, the animals were examined daily for clinical signs of illness.

Hematologic studies were conducted on 2 of the animals during the course of the infection to detect the presence or absence of changes in cellular elements and in the blood clotting mechanism. Red and white blood cell counts and differential white blood cell counts were performed in the usual manner. Blood coagulation times were measured as outlined by Haden.[3] Clot retraction was taken as indirect evidence of blood platelet function. Prothrombin times were determined according to the method of Quick.[5]

Gross and microscopic examinations were made of the tissues of each animal. Necropsies were performed either immediately after the infections terminated in death or after euthanasia with chloral hydrate. Histopathologic examinations were made on formalin-fixed, paraffin-embedded tissue sections and stained with hematoxylin and eosin. On some occasions, a special connective-tissue stain, Mallory's phosphotungstic acid–hematoxylin,[4] was used to identify tissue components.

RESULTS

The gross postmortem findings in deer experimentally infected with the EHD viral isolates agree with those reported by Shope for the deer which he examined.[8] The extensive hemorrhages which occurred in every deer led to the suspicion that a deranged clotting mechanism might be partly responsible.

Clinical Findings.—In both animals on which hematologic studies were performed, it was found that the clotting time was prolonged. In 1 deer this change progressed from the normal clotting time of 3 to 5 minutes to a clotting time of 23 min-

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Paper NS-280 from the Department of Veterinary Science, University of Wisconsin (Karstad and Winter) and the Wisconsin Conservation Department (Trainer), Madison. Dr. Winter is now on the staff of the Department of Veterinary Science, Pennsylvania State University, University Park.

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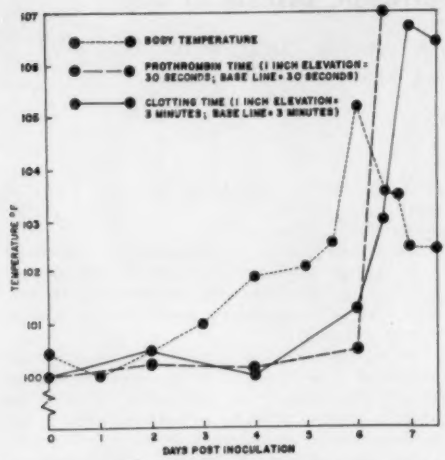
BODY TEMPERATURE
PROTHROMBIN TIME (1 INCH ELEVATION = 30 SECONDS; BASE LINE = 30 SECONDS)
CLOTTING TIME (1 INCH ELEVATION = 3 MINUTES; BASE LINE = 3 MINUTES)
TEMPERATURE °F
DAYS POST INOCULATION

Fig. 1—Body temperatures, prothrombin times, and clotting times recorded throughout the illness of a deer inoculated with the South Dakota strain of epizootic hemorrhagic disease of deer.

utes, just before death. This increase in blood coagulation time was somewhat parallel to the elevation in body temperature.

Prothrombin time also was markedly increased. This was observed first about the sixth day postinoculation, at which time body temperature became greatly elevated. Clot retraction also became progressively poorer during the course of the infection. These changes are summarized (fig. 1, 2).

Bleeding time was greatly prolonged during the latter stages of the disease. This occurred to the extent that blood was seen to drip from the jugular puncture wound, made by an 18-gauge needle, for up to 30 minutes after a blood sample was taken.

Total red blood cell counts changed little during the course of the infection. Slightly elevated counts near the end of clinical illness may have signified hemoconcentration. In both animals examined, it was observed that the percentage of lymphocytes decreased and the percentage of neutrophils increased during the latter stages of the infection, while total white blood cell counts remained about the same. This information, with the body temperature and blood coagulation values, is summarized (tables 1, 2).

Fever usually appeared first on the fifth or sixth day postinoculation. Body temperatures rose sharply to about 105 F., then decreased more or less gradually until death within the next 2 to 5 days.

Animals ceased to eat and rarely drank after the onset of fever. Their natural alertness persisted until they were *in extremis*, at which time they reacted poorly to external stimuli. Finally, just before death, they entered a shock-like state marked by extreme weakness, rapid heart rate, and labored breathing with an expiratory grunt. Dribbling of blood-stained urine occurred in every animal at or just before death. Bloody feces often were observed, or blood was found in the rectum when temperatures were taken.

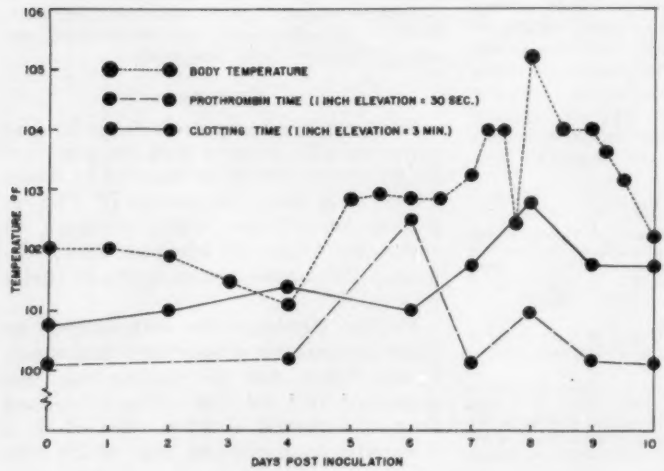
BODY TEMPERATURE
PROTHROMBIN TIME (1 INCH ELEVATION = 30 SEC.)
CLOTTING TIME (1 INCH ELEVATION = 3 MIN.)
TEMPERATURE °F
DAYS POST INOCULATION

Fig. 2—Body temperatures, prothrombin times and clotting times recorded throughout the illness of a deer inoculated with the New Jersey strain of epizootic hemorrhagic disease of deer.

TABLE 1—Body Temperature and Hematologic Values Obtained on a Deer Inoculated with the Virus of Epizootic Hemorrhagic Disease (EHD), South Dakota Serotype

Days postinoculation	Body temperature (F.)	Prothrombin time (min.)	Clotting time (min.)	r.b.c. (Millions/cmm)	w.b.c. Total (thousands/cmm.)	Lymphocytes (%)	Monocytes (%)	Neutrophils (%)	Eosinophils (%)	Basophils (%)
0	100.4	0.5	3.0	10.8	2.6	62	1	34	3	0
1	100.0	....	....	.....	....	....	..	....	..	..
2	100.5	0.6	4.5	9.9	2.6	65	1	25	10	0
3	101.0	....	....	.....	....	....	..	....	..	..
4	101.8	0.5	3.0	9.5	2.7	71	1	24	3	1
5	102.6	....	....	.....	....	....	..	....	..	..
6	105.2	0.8 (a.m.) 4.+(p.m.)	7.0 (a.m.) 12.0 (p.m.)	9.8	2.5	24	1	75	0	0
7*	102.4	4.+	23.0	11.1	2.5	19	1	80	1	0

* Animal died on the seventh day postinoculation.

Pathologic Findings.—Hemorrhages, both gross and microscopic, were present in virtually every organ and tissue of the body. The fresh appearance of the blood in these hemorrhages indicated that they must have occurred in the later stages of the illness. In some deer, hemorrhages of large size produced infarct-like lesions in the heart or kidneys. In 2 deer, dissecting hemorrhages were found in the walls of the aorta (fig. 3). Large hemorrhages into the apical myocardium were seen, and extensive hemorrhage into the musculature of the atria was a constant finding. In some instances, the atria were almost black due to the quantity of extravasated blood. In 1 deer there were massive hemorrhages into the perirenal tissues. Another deer was found to have a large hemorrhage involving the mesenteric vessels to the small intestine, so that at necropsy the appearance was similar to acute intestinal strangulation with congestion, hemorrhage, and necrosis of a 24-inch segment of intestine. Petechial and ecchymotic hemorrhages were found on all serous and mucosal surfaces.

The immediate cause of death varied from animal to animal, depending upon the distribution of the most severe lesions. One deer experienced extreme respiratory difficulty just before death. At necropsy, this deer was found to have extreme pulmonary edema and emphysema. The bronchi and trachea were filled completely with blood-stained froth, the epithelium of the trachea was covered with petechial hemorrhages, and the subepithelial connective tissues of the trachea and interlobular connective tissues of the lungs were markedly infiltrated by a gelatinous transudate.

Serogelatinous transudates were seen in the subcutis and in the fascial planes of the skeletal muscles of most of the animals. Pericardial and pleural fluids were generally increased in amount, sometimes blood-tinged.

The most pronounced brain lesions were perivascular hemorrhages and edema. De-

TABLE 2—Body Temperature and Hematologic Values Obtained from a Deer Inoculated with the Virus of Epizootic Hemorrhagic Disease (EHD), New Jersey Serotype

Days postinoculation	Body temperature (F.)	Prothrombin time (min.)	Clotting time (min.)	r.b.c. (Millions/cmm.)	w.b.c. Total (thousands/cmm.)	Lymphocytes (%)	Monocytes (%)	Neutrophils (%)	Eosinophils (%)	Basophils (%)
0	102.0	0.3	2.0	10.2	4.3	64	3	27	1	5
1	102.0	....	....	.....	....	....	..	....	..	..
2	101.9	....	3.0	.....	....	....	..	....	..	..
3	101.5	....	....	.....	....	....	..	....	..	..
4	101.1	0.5	4.0	9.9	4.3	81	0	16	3	0
5	102.8	....	....	.....	....	....	..	....	..	..
6	102.8	2.5	3.0	7.4	2.6	....	..	....	..	..
7	104.0	0.5	5.0	7.4	4.4	49	1	47	3	0
8	105.2	0.9	8.0	8.0	2.4	49	0	47	2	0
9	103.2	0.5	5.0	9.9	2.4	39	0	60	1	0
10*	102.2	0.5	5.0	13.1	2.4	53	0	47	0	0

* Euthanasia performed on the tenth day postinoculation.

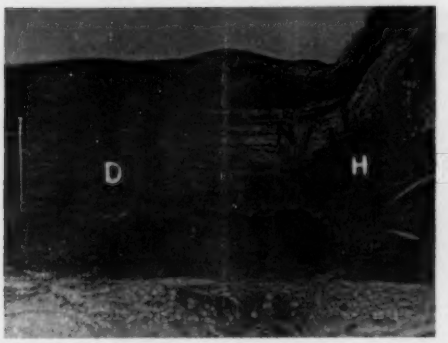

Fig. 3—Aorta of deer with epizootic hemorrhagic disease. Large dissecting hemorrhage (H) surrounded by zones of necrosis (N) and degeneration (D); perivascular hemorrhage into the adventitia is also present (P). H & E stain; x 24.

Fig. 4—Lymph node of deer with epizootic hemorrhagic disease showing "reaction centers"; germinal centers are devoid of lymphoid tissue and contain increased amounts of collagen. H & E stain; x 67.

generative changes observed in nerve cells were probably due to interrupted blood supply. Periventricular edema was pronounced in the cerebral hemispheres of 1 deer. The only signs of inflammation were small collections of mononuclear leukocytes around some of the meningeal blood vessels.

The microscopic vascular changes, which were seen throughout the body and which appeared to be associated with the hemorrhages, consisted mainly of over-dilation of vessels with degeneration and necrosis of smooth muscle and adventitia. Arterioles and venules were most markedly affected. Signs of inflammation were usually absent. The endothelium appeared in general to be little affected, although in many deer, owing to the pressure of the blood and lack of support from the degenerated muscle and connective tissue coats, it was greatly dilated or ruptured.

Lymphatic organs throughout the body were hemorrhagic and partially depleted of lymphatic tissue. Lymph nodes, which were grossly enlarged, edematous and hemorrhagic, contained follicles greatly reduced in size. The germinal centers had the appearance of "reaction centers" in which lymphocytes were few or absent, leaving only the reticular tissue framework and increased amounts of collagen (fig. 4). Necrosis of lymphatic tissue in germinal centers was observed in one instance. Edema and hemorrhages were most prominent in the medullary portions of the nodes. In many nodes, the extravasated red blood cells were found to have been engulfed by macrophages but were usually intact. Only hemorrhages of the longest duration had any evidence of blood-cell lysis.

Similar changes were seen in the spleen. This organ, which in some deer appeared to be affected with acute splenic tumor, was found to be acutely engorged with blood so that it was difficult to make good histologic sections. Lymphoid follicles were markedly reduced in size. Depletion of white pulp throughout the organ allowed the network of smooth muscle fibers to show through. In 1 deer, the trabeculae were fragmented and there was evidence of degeneration and necrosis of their smooth muscle and connective tissue elements.

Gross changes in the lungs consisted of congestion, generalized edema, and emphysema. On microscopic examination, the vascular changes previously described were found to be pronounced and were associated with small hemorrhages into the alveoli. Emphysema was probably compensatory, the result of edema and dyspnea just before death. Hemorrhages into the subepithelial tissues of the bronchi and trachea were seen in 2 deer.

Pathologic changes were seen in the kidneys of all deer examined. The renal lesions most consistently observed were ex-

Fig. 5—Kidney of deer with epizootic hemorrhagic disease; glomerulus and proximal collecting tubules contain proteinaceous material. Some cells of the tubular epithelium are necrotic. H & E stain; x 126.

treme congestion and necrosis of convoluted tubules. Extensive bilateral cortical necrosis was seen in 1 deer. Also prominent were the vascular degeneration and necrosis observed in other organs. Hemorrhages were found throughout the cortical and medullary regions in 2 of the 6 deer. In 2 deer there were focal infiltrations with mononuclear leukocytes in the cortex and at the corticomedullary junction. Large hyaline casts in the collecting tubules were present in the kidneys of 1 deer. In some deer, glomeruli and proximal collecting tubules were seen to contain proteinaceous material (fig. 5).

Liver lesions were few and consisted chiefly of congestion and atrophy of cells bordering the distended sinusoids. Mild degenerative changes, such as fatty metamorphosis of hepatic cells, were seen in every deer. In only 1 deer were areas of focal necrosis observed. These seemed to be associated with, and possibly the result of, small hemorrhages into the liver parenchyma. Infiltrations of leukocytes, mostly mononuclear cells, were found in the portal triads of 4 of the 6 livers examined.

The adrenal glands were congested in all animals. In 1 deer, hemorrhages into the cortex and medulla were associated with areas of focal necrosis.

Pancreatic lesions were limited to congestion, hemorrhages, and the previously described vascular degeneration and necrosis.

In addition to the hemorrhagic lesions, the myocardia of all deer contained areas of degeneration and necrosis of the muscle and connective tissue elements (fig. 6). In 1 deer, a large infarct was ascribed to the presence of a large apical hemorrhage. In 3 of the animals there was evidence of myocarditis in the form of focal areas of mononuclear leukocyte infiltration. In most deer, the endocardium also was involved, showing thickening and fibrinoid degeneration of the supporting connective tissue, subendocardial hemorrhage, leukocyte infiltration, and, in 1 deer, erosion of the endocardium. The changes in the pericardium consisted chiefly of hemorrhages, edema, fibrinoid connective tissue degeneration, and leukocyte infiltration.

In all instances, there was aortic degeneration, necrosis of the media, and degeneration of the adventitia associated with perivascular hemorrhages. Two of the animals had dissecting hemorrhages of the aorta (fig. 3).

In these young deer, there was extreme congestion, edema, vascular necrosis, and hemorrhages in the thymus which was not yet involuted. Edema and degeneration were prominent in the fibrous supporting tissues of the gland.

The testicles were examined histologically in only 3 deer. In all instances, edema and focal hemorrhages were associated with

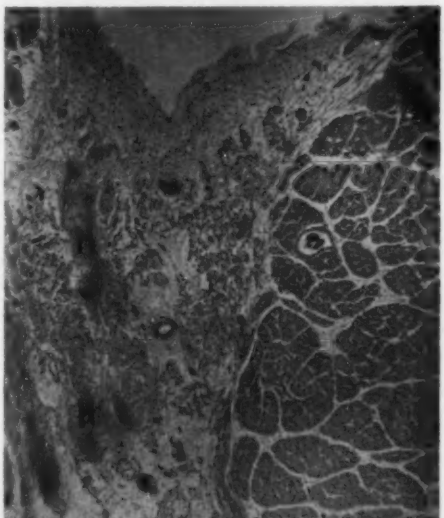

Fig. 6—Heart of deer with epizootic hemorrhagic disease; there is hemorrhage (H) into an area of necrotic myocardium; normal myocardium is on the right. H & E stain; x 32.

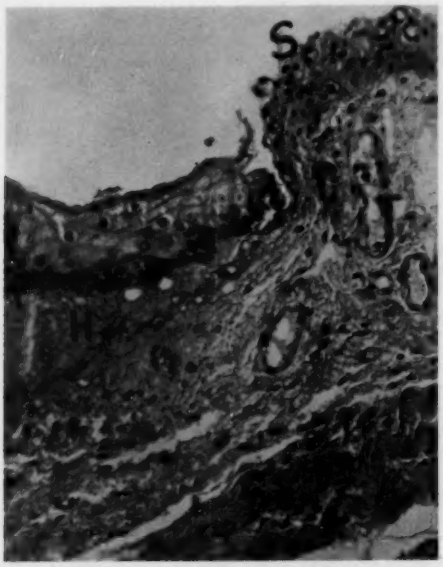

Fig. 7—Reticulum of deer with epizootic hemorrhagic disease; subepithelial hemorrhage (H) has lead to sloughing of the epithelium (S). H & E stain; x 185.

necrosis of the germinal epithelium and supporting connective tissues.

The lesions in the gastrointestinal tract were similar to those described for other portions of the body and consisted mainly of hemorrhages in all tissue layers, associated with degeneration and necrosis of blood vessel walls. The connective tissue elements, submucosal, intermuscular, and subserosal, were considerably swollen and edematous. In 1 deer, acute obstruction of blood flow due to massive hemorrhage was found to have produced total necrosis of a 24-inch segment of intestine. Epithelial sloughs occurred in localized areas throughout all portions of the stomach and intestinal tract (fig. 7). In 1 deer, these were apparently of sufficient duration to be typical ulcers. The abomasum and intestine contained focal infiltrations of mononuclear leukocytes in the submucosal and subserosal connective tissue layers. In 1 animal, large numbers of eosinophils were seen in the intestinal submucosa. Submucosal lymph follicles evidenced the same degree of depletion of germinal centers as was seen in the lymph nodes.

In all the deer examined, smooth and striated muscles were edematous and hemorrhagic with degeneration and necrosis of muscle fibers (fig. 8). There was swelling, loss of striations, and hyalinization of degenerated voluntary muscle fibers. Focal areas of leukocyte infiltration were present in the skeletal muscles of 1 deer (fig. 9).

Fig. 8—External muscle layer of the abomasum of deer with epizootic hemorrhagic disease; there is vacuolation and dissolution of smooth muscle fibers. H & E stain; x 185.

The skin on varied parts of the body was found to contain gross and microscopic hemorrhages. Histologic examination of the subcutaneous connective tissue and muscles revealed scattered areas of degeneration and necrosis (fig. 10).

Sternal marrow, examined in the 2 deer in which hematologic studies were made, appeared relatively normal. In these 2 deer, megakaryocytes were found to be fewer in the marrow of the deer which experienced the most severe illness. These differences, however, compared with what is presumed to be normal, were not sufficient to suggest a deficiency of the platelet-forming elements.

Discussion and Conclusions

The occurrence of hemorrhages associated with vascular lesions was found almost uniformly in all 6 experimentally infected animals. In this regard, EHD virus of the New Jersey, South Dakota, and Michigan strains appeared to produce the same pathologic changes. The extensive character of the hemorrhages in the first 3 animals infected suggests the possibility of derangement of the normal blood clotting mechanisms. Therefore, observations on the fourth and fifth animals infected were extended to include measurement of clotting time, prothrombin time, clot retraction, and counts of the formed elements of the blood. This information (fig. 1, 2; tables 1, 2) indicates that blood coagulation time and prothrombin time became greatly prolonged and that this increase roughly paralleled that of body temperature.

Measurements of prothrombin time were carried out on 0.4 per cent citrated blood which had been stored for 1 to 6 days at 4 C. Therefore, these values may not be accurate. However, it is felt that the pronounced prolongation in prothrombin time observed during the latter stages of the illness indicates a trend and, therefore, it may be taken as evidence of reduced blood prothrombin activity (fig. 1). The deer inoculated with the New Jersey strain was less acutely infected and also had less reduction in prothrombin activity (fig. 2). It is possible that this animal, if not killed, would have recovered from the disease.

The poor clot retraction, as observed during the clinical stage of infections, is usually taken to indicate a deficiency of blood platelets. Platelet counts were not made. According to Quick,[5] the presence of megakaryocytes in normal numbers in the bone marrow gives no assurance that blood platelets are present in suitable numbers.

It was not determined that the bone marrow was depleted in the 2 deer examined.

Reduced prothrombin activity seems to be an adequate explanation for the prolonged clotting time. Quick[5] stated that liver lesions are the most common cause of hypoprothrombinemia. Changes in hepatic cells were consistently found in the experimentally infected animals. These were generally limited, however, to mild degeneration or atrophy of cells bordering dilated sinusoids. Nevertheless, in the 1 deer in which areas of focal hepatic necrosis were seen, the prothrombin content was greatly reduced (fig. 1). Likewise, in the other animal in which it was possible to compare liver lesions with prothrombin content, there were only mild liver changes with much less prolongation of prothrombin time (fig. 2). The impaired blood coagulation no doubt facilitates the escape of blood from damaged vessels.

The total red and white blood cell counts obtained from the 2 deer examined were within the limits reported by others in studies on apparently normal deer.[6,9] A point of interest is the increase in percentage of neutrophils and the decrease in percentage of lymphocytes observed during the latter stages of the infection (table 1, 2). It is

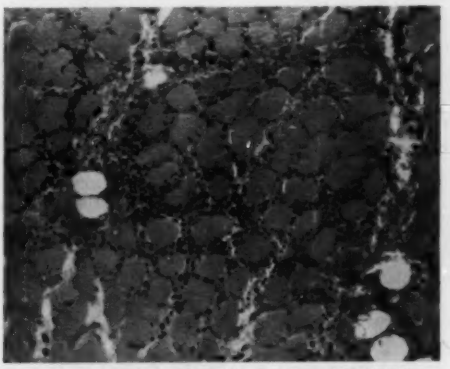

Fig. 9—Skeletal muscle of deer with epizootic hemorrhagic disease containing hemorrhages and mononuclear leukocytic infiltrates between the muscle fibers. H & E stain; x 100.

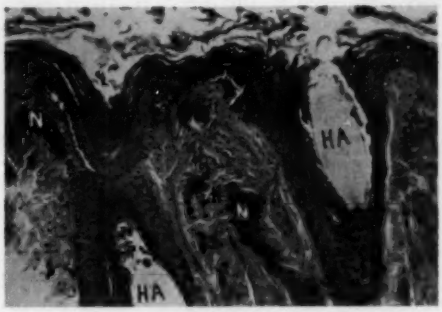

Fig. 10—Skin of deer with epizootic hemorrhagic disease. Areas of necrosis (N) and hemorrhage (H) are present in the subcutaneous muscles and connective tissue. Two hairs (HA) are included in the photograph. H & E stain; x 84.

probable that these changes were the result of stress accompanied by increased release of adrenocorticosteroids.

The partial depletion of lymphatic tissues throughout the body may be related to stress, or it may represent direct viral action on these tissues.

The means by which degeneration and necrosis of the muscle and connective tissue of blood vessel walls occurred is unknown. The primary cause may have been overdilatation as the result of a toxin liberated from damaged tissues. Or, the observed degenerative changes may have been the direct result of viral action upon cells. If so, this would represent a viral tropism for tissues of mesenchymal origin. Another explanation for these changes, as well as for the degeneration and necrosis of striated and cardiac muscle, may be that these tissues were damaged as the direct result of interrupted blood supply, which in turn was caused by the hemorrhages observed. This is probably the way in which the large infarct-like lesions were produced in the heart, intestines, and kidneys.

An interesting observation is the almost complete absence of signs of inflammation in the tissues of the deer studied. Inflammatory changes were limited to slight leukocyte infiltration in the heart and skeletal muscles, the submucosal and intermuscular connective tissues of the stomach and intestines, the interlobular connective tissue of the liver, and the adventitia of the blood vessels.

These leukocytes were mostly mononuclear cells, although a few neutrophils were seen in some areas. The infiltrations of eosinophils seen in the intestinal submucosa of 1 deer may have been due to parasitism. Examinations for helminth or protozoan parasites were not made.

SUMMARY

Six deer were infected experimentally with the virus of epizootic hemorrhagic disease (EHD) of deer to study the pathologic changes produced and the mechanism by which these changes arose.

Hematologic studies revealed that a prolonged blood coagulation time was related to prothrombin deficiency. This derangement of the blood clotting mechanism, plus demonstrated degenerative changes in blood vessel walls, resulted in multiple microscopic to massive hemorrhages. Most vessels which were not ruptured appeared to be greatly dilated, explaining the shock seen just before death.

Multiple hemorrhages produced a variety of degenerative changes in the associated tissues and organs. These changes were interpreted, at least partially, to be the result of an interrupted blood supply.

The characteristic clinical response to the infections was a sharp rise in body temperature at about 5 days postinoculation, with anorexia and weakness. This was followed by gradual depression of body temperature, associated with a shock-like condition, until death occurred 2 to 6 days later.

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SUMMARIO IN INTERLINGUA

Pathologia de Epizootic Morbo Hemorrhagic de Cervos

Sex cervos *(Odocoileus virginianus)* esseva inficite experimentalmente con le virus de epizootic morbo hemorrhagic de cervos pro studiar le alterationes pathologic resultante e le mechanismos per que ille alterationes se produceva.

Studios hematologic revelava que le occurrentia de un prolongate tempore de coagulation del sanguine esseva relationate a un deficientia de prothrombina. Iste perturbation del mechanismo coagulatori del sanguine, insimul con demonstrate alterationes degenerative in le parietes del vasos de sanguine, resultava in multiple hemorrhagias de grados inter microscopic e massive. Le majoritate del vasos que non esseva rupturate exhibiva un apparentia de forte dilatation, lo que explica le choc que es a observar immediatemente ante le morte.

Multiple hemorrhagias produceva un varietate de alterationes degenerative in le associate tissus e organos. Iste alterationes esseva interpretate, al minus in parte, como resultato de un interrumpite provision de sanguine.

Le characteristic responsa clinic al infectiones esseva un acute augmento del temperatura del corpore a un periodo de circa cinque dies post le inoculation. Isto esseva associate con anorexia e debilitate e sequite per un depression gradual del temperatura del corpore que progredeva usque al momento del morte e que esseva associate con un condition simile a choc le qual superveniva inter duo e sex dies plus tarde.

Electron Microscopy of Infectious Canine Hepatitis Virus Grown in Cultures of Canine Kidney Cells

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THE NATURE of intranuclear inclusion bodies associated with viral lesions has long been a subject of controversy. Although the technique of ultrathin sectioning has given much valuable information on the host cell-virus relationship, there are still many questions to be solved as to the submicroscopic structure of intranuclear inclusion bodies. The most difficult point in studying these problems seems to be that the inclusion bodies are not always identifiable by electron microscopic study of thin sections of infected tissues. In an earlier electron microscopic study, 2 workers [30] reported that, in the hepatic parenchymal cells of pups affected with infectious canine hepatitis (ICH), the nuclei contained numerous viral particles at locations apparently corresponding to the sites of inclusion bodies seen with a light microscope.

This work was undertaken to elucidate the mode of development of the inclusion bodies and viral particles by observing, with light and electron microscopes, cells taken at specified times after inoculation and to correlate these morphologic changes with the virus release determined by infectivity titrations.

It was desirable to infect all the cells nearly simultaneously, and to synchronize morphologic changes developed in them so that each sample would uniformly represent a definite stage of infection. To fulfill these conditions as much as possible, monolayer cultures of canine kidney epithelium were used as a host cell system, since it has been reported by Cabasso *et al.*,[6] Fieldsteel and Emery,[11] and Leader [17] that ICH virus multiplies readily in such a culture system and causes distinctive cytologic changes.

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MATERIALS AND METHODS

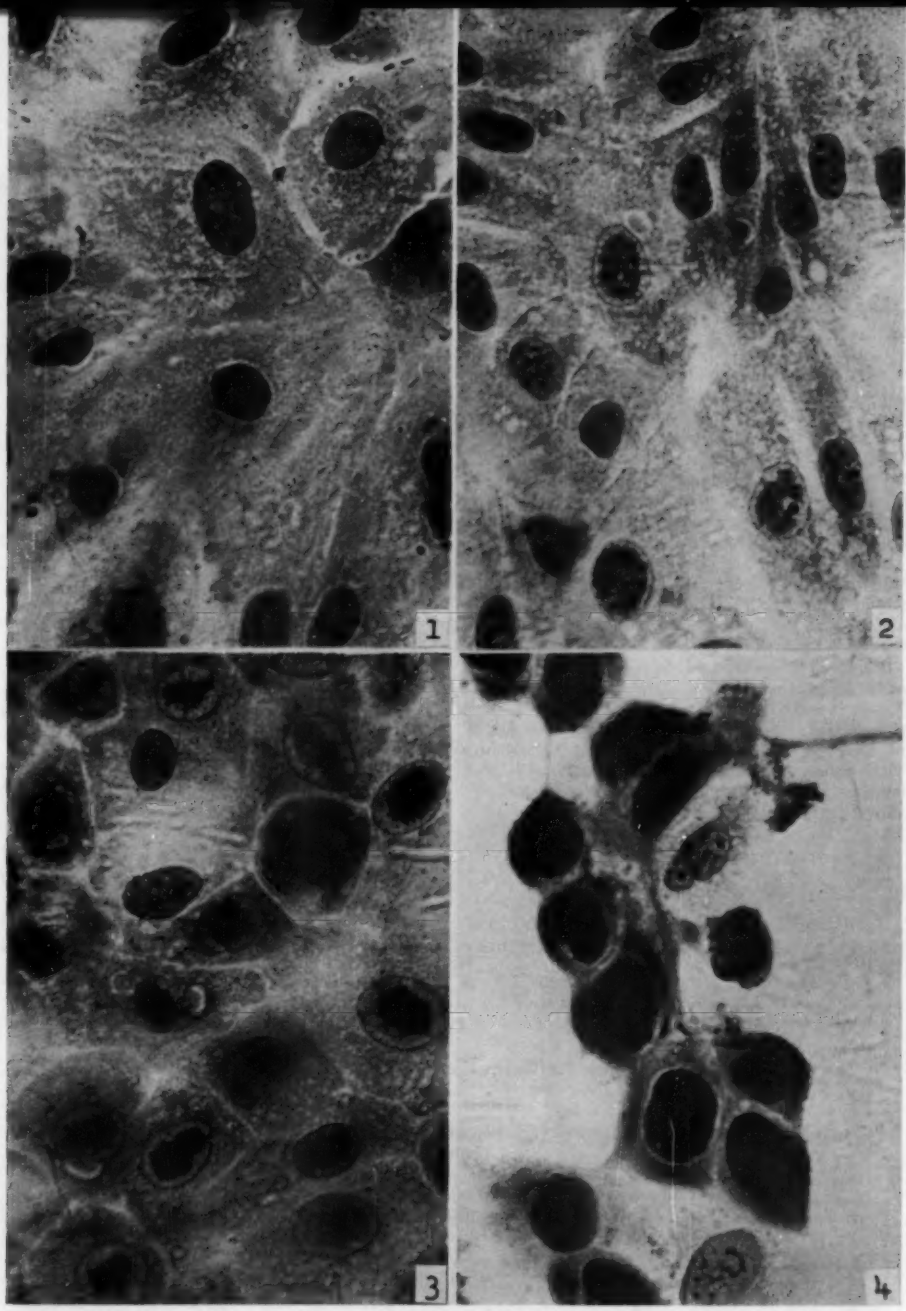
Virus Strains.—Two strains of ICH virus were used. One was the D-43 strain, which was the same as employed previously,[30] and the other was the Matsuda strain, furnished by Professor K. Hirato, Hokkaido University. The source of virus for the present work was the infective fluid phase, harvested from the third tissue-culture passage of each strain. The 50 per cent tissue culture infective doses ($t.c.i.d._{50}$) of these materials were between $10^{6.5}$ and $10^{7.5}$ per milliliter.

Tissue Culture.—Monolayer cultures of kidney epithelial cells from young mongrel dogs were prepared. For morphologic examination, trypsinized cells were propagated on the wall of a 15- by 150-mm. test tube containing a 6- by 18-mm. cover slip. Each culture was started with 1.0 ml. of 1:200 cell suspension. The culture medium consisted of 0.5 per cent lactalbumin enzymatic hydrolysate, 5 per cent horse serum, and 94.5 per cent Hanks' balanced salt solution containing 100 units of penicillin and 100 μg. of streptomycin per milliliter.

Infection of Cultures.—After standing in a stationary position for 4 to 6 days at 37 C., the medium in the culture tubes with developing monolayer cell sheets was renewed with 1.0 ml. of fresh medium in which 10^{-3} of the virus material was suspended. Two hours after inoculation, the cultures were washed 5 times successively with Hanks' balanced salt solution, and fresh nutrient medium was added. The inoculum, as well as the medium, collected just after washing was stored at −20 C. for virus assay.

Sampling and Titration.—Cover slips were removed from every 4 tubes at 6, 12, 15, 18, 24, and 48 hours following inoculation. Two of them were placed in fixative for light microscopy and 2 were used for electron microscopy. The fluid of these cultures was pooled and stored at −20 C. for the titration of infective virus. Uninoculated cultures were similarly harvested at 12, 24, and 48 hours after commencement of the experiment and were used as controls for possible nonspecific cellular changes. Virus titers were measured by making tenfold dilutions of the material in the nutrient medium. After that, 2 tubes containing canine kidney cells were inoculated with 1.0 ml. of each dilution. The inoculated cultures were examined for 7 days and the end points of virus infection presenting cytopathic effects were expressed as $t.c.i.d._{50}$.

Preparation of Specimens for Electron Microscopy.—Methods for cutting thin sections and use



Materials shown in figures 1 to 4 were fixed in absolute methanol and stained according to Giemsa's method.

Fig. 1.—Uninfected tissue culture cells of canine kidney epithelium. x 600.

Fig. 2—Eighteen hours after inoculation with the D-43 strain. Almost all cells contain an intranuclear inclusion body consisting mainly of several irregular eosinophilic granules. x 600.

Fig. 3—Twenty-four hours after inoculation with the D-43 strain. Most cells contain a single, large, dark inclusion body near the center of the nucleus. x 600.

Fig. 4—Forty-eight hours after inoculation with the D-43 strain. In practically every cell there is severe karyopyknosis, and it is difficult to observe an intranuclear inclusion body. x 600.

of the electron microscope were essentially the same as described for the previous work.[20] After removal from the culture tubes, the cover slips and adhering cells were washed in balanced salt solution and transferred, for fixation, to 1 per cent osmium tetroxide buffered at pH 7.4, as recommended by Palade.[25] After fixation for 20 minutes, the cells adhering to the cover slips were rapidly dehydrated in 70 per cent, 95 per cent, and absolute ethanol, then transferred to a mixture of 2 parts methyl and 8 parts butyl methacrylate. For final embedding in a capsule, the cell sheet was detached gently from the cover slip with a razor blade and placed on the bottom of a gelatin capsule. Then the capsule was filled with the same methacrylate mixture, to which 2 per cent of benzoyl peroxide had been added as catalyst. Polymerization was carried out at 45 C.

Light Microscopy.—After removal from the culture tubes, the cover slips were placed in absolute methanol or Bouin's fluid to fix the adhering cells and then stained with hematoxylin and eosin or by Giemsa's method.

The tissue blocks used for electron microscopy were also examined by the light microscope. Adjacent, relatively thick sections were taken when thin sections were cut, so that the same cells might be observed by both electron and light microscopy. The sections were mounted on glass slides, the methacrylate being removed with toluene, and examined after staining with Giemsa solution.

RESULTS

MORPHOLOGIC OBSERVATIONS

Control Cultures.—In stained preparations, normal cells appeared similar to those described in detail by Leader.[17] Their pale cytoplasm appeared homogeneous or finely granular and sometimes contained vacuoles with variable diameters. The nucleus had a fine chromatin network and contained 1 to 4 dark, well-defined nucleoli (fig. 1).

An electron micrograph of such a cell, sectioned almost vertically to the cell sheet, is shown (fig. 5). The nucleus appeared oval or rounded but with occasional indentations. The nuclear membrane was of uniform thickness, and grainy nuclear chromatin was evenly and finely dispersed. One or more dense nucleoli were seen in many of the sections. Mitochondria were relatively small and widely dispersed throughout the cytoplasm. Many circular and elongated profiles of the endoplasmic reticulum were scattered throughout the cytoplasm, and occasionally osmiophilic lipid inclusions were also seen.

Infected Cultures.—The results obtained from experiments with the 2 virus strains (D-43 and Matsuda) were essentially the same, with the exception that in the latter, as will be described, viral particles often formed intranuclear crystalline aggregates in infected cells.

In cells of cultures harvested 6, 12, and 15 hours after inoculation, no changes were present which could be morphologically related to inclusion body or viral particle formation, though a large number of cells

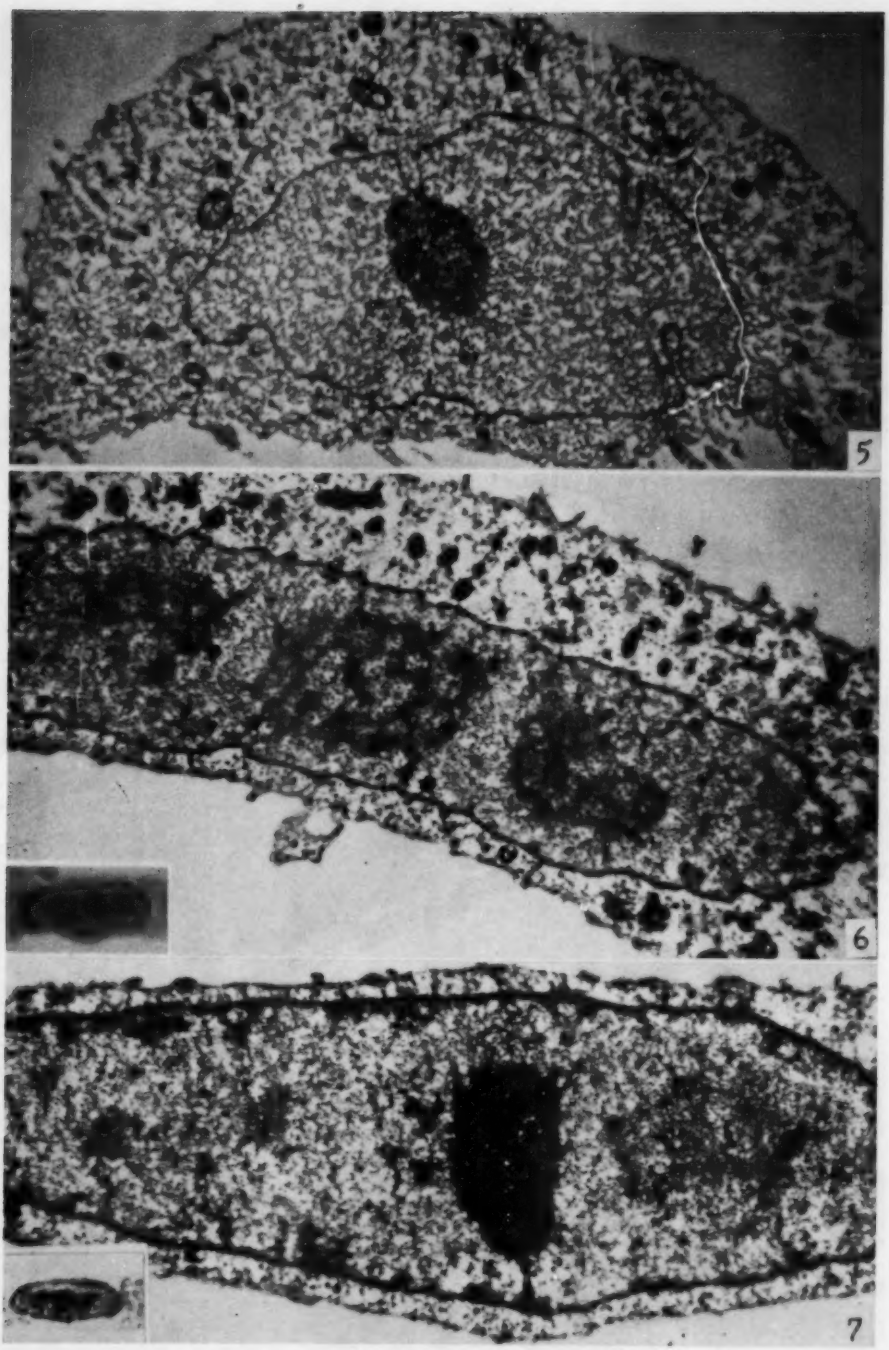
Legends for Illustrations on Opposite Page

Figures 5 to 7 are electron photomicrographs of sections from which no embedding plastic was removed.

Fig. 5—A normal cell of canine kidney epithelium from an uninoculated tissue culture. Near the center of the figure is the nucleus with its dense nucleolus and a few indentations. Nuclear chromatin is finely and evenly dispersed. Several mitochondria and many circular and elongated profiles of the endoplasmic reticulum are scattered throughout the cytoplasm. x 8,960.

Fig. 6—Eighteen hours after inoculation with the D-43 strain. A nucleus with 3 irregular-shaped masses of dense, finely granular material. The chromatin pattern is not notably altered. No viral particles are visible in this cell. x 8,700. Inset shows the same cell observed in an adjacent thick section stained by Giemsa's method. Three eosinophilic granules in the nucleus appear to correspond, in their shape and position, to the irregular masses of dense, finely granular material seen in the electron micrograph. x 1,350.

Fig. 7—Part of a cell 18 hours after inoculation with the D-43 strain. A prominent nucleolus composed of dense, fine granules is present near the center of the nucleus. On either side of the nucleolus are seen irregular-shaped masses of finely granular material of somewhat lower density than those constituting the nucleolus. The chromatin pattern is slightly disturbed and small clumps of chromatin adhere to the nuclear membrane. A small number of viral particles are scattered throughout the nucleus. x 14,000. Inset shows similar nuclear alterations of the same cell seen in a section cut at a slightly different level and stained by Giemsa's method. x 1,350.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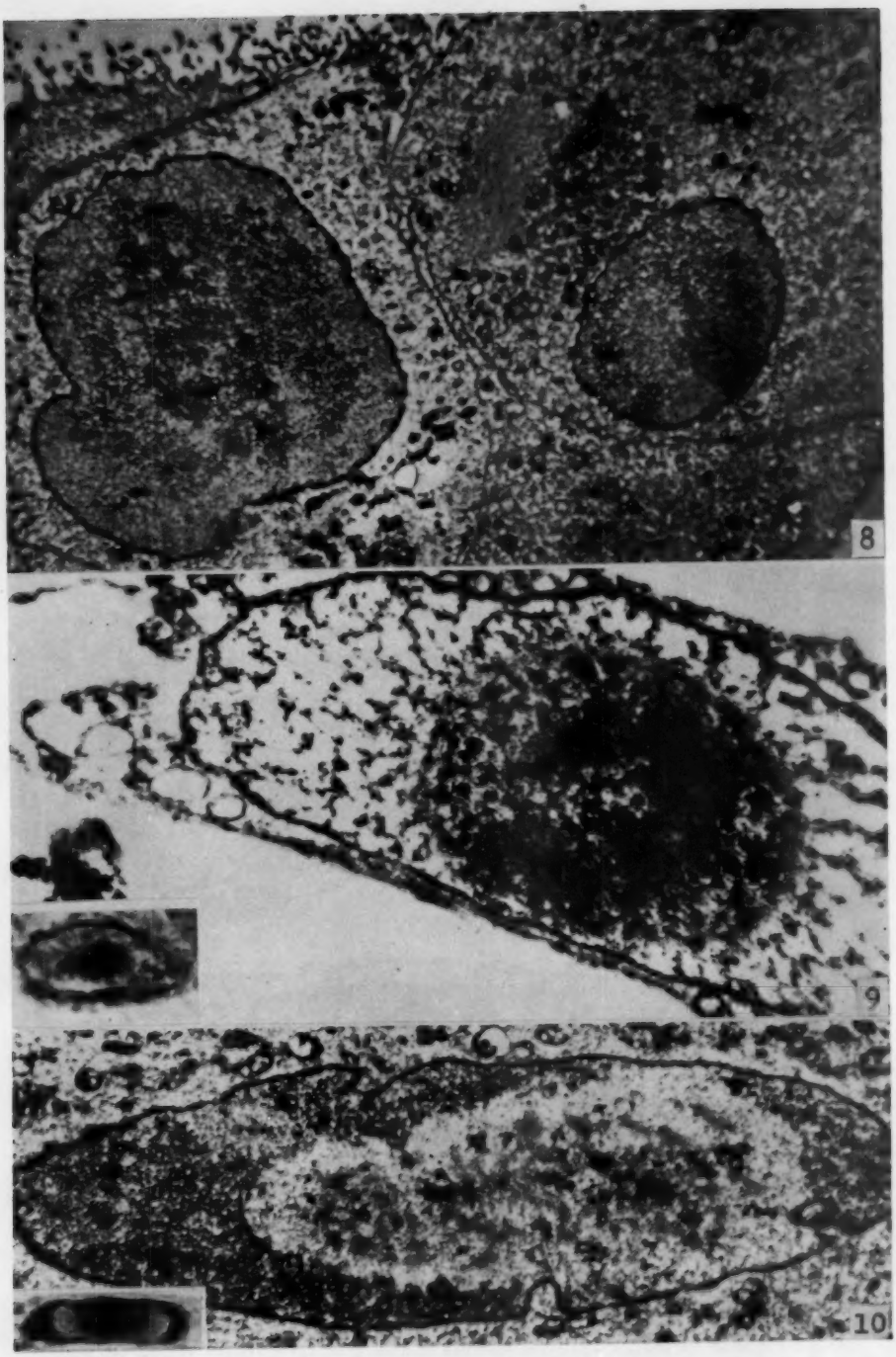
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were examined under both the light and electron microscopes. In the majority of cells studied, intranuclear inclusion bodies were first clearly seen 18 hours following inoculation. In stained preparations, these inclusion bodies were generally composed of several irregular, eosinophilic, granular masses (fig. 2). The nucleoli intervened between granular masses or were displaced to the periphery of the nucleus. As seen in electron micrographs, the earliest typical nuclear changes were characterized by the presence of multiple, reticulated condensations of dense, fine granules. These condensations of the finely granular material were irregular in shape and varied in size; they occurred at random in the nucleus and apparently corresponded to the eosinophilic, granular masses seen by the light microscope in the adjacent thick section (fig. 6, 7). At this stage of infection, the majority of the nuclei contained masses of dense, finely granular material but no recognizable viral particles. In some of these nuclei, the chromatin pattern was similar to that of the uninfected control cells (fig. 6). In others, chromatin decreased to irregular clumps, and a small number of viral particles were seen in close spatial relationship to the finely granular material (fig. 7). In both types, the cytoplasm was usually well preserved.

Twenty-four hours following inoculation, though the sheet of cells remained intact, the majority of cells contained a single large inclusion body near the center of the nucleus (fig. 3). The inclusion bodies were stained darker and more homogeneously than those observed in 18-hour cultures and were often surrounded by a clear zone. In some nuclei, there were margins of chromatin. Thus, some of the inclusion bodies at this stage represented typical examples of the type A inclusion body of Cowdry.[7] In low-power electron photomicrographs, the majority of the nuclei had a large central mass consisting of numerous viral particles and dense, finely granular material which appeared to correspond to an inclusion body observed by the light microscope (fig. 8, 9). However, in some nuclei which appeared to be at advanced stages of morphologic changes, no clearly outlined central masses could be observed. In these nuclei, the finely granular material decreased in amount and the viral particles were scattered throughout the nucleus. The viral particles tended to be dispersed toward the nuclear periphery and to accumulate at the inner aspect of the nuclear membrane. Moreover, it should be emphasized that another type of nuclear alteration was repeatedly encountered. In this type, only a few viral particles were visible in the central mass; whereas, numerous particles appeared to develop in the granular material accumulated in the peripheral zone of the nucleus (fig. 10).

From 36 to 48 hours after inoculation, most cells had marked cytopathic changes and began to detach from the glass surface. In such cells, it was difficult to observe an

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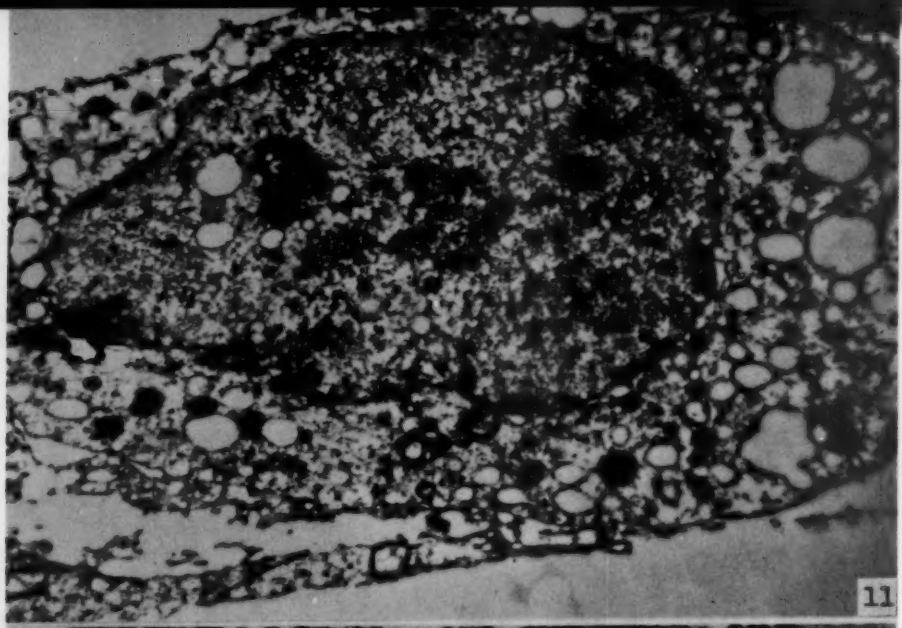
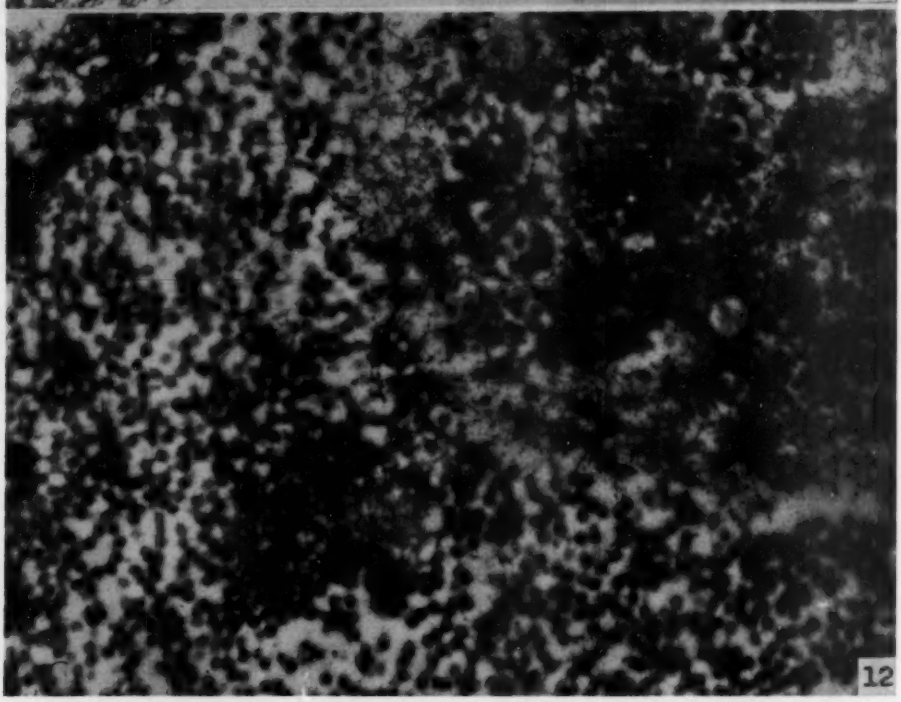
Figures 8 to 10 are electron photomicrographs of material from which no embedding plastic was removed.

Fig. 8—Twenty-four hours after inoculation with the D-43 strain. Two nuclei containing a large central mass of numerous viral particles and of a relatively small amount of finely granular material, respectively. In them, the nuclear membrane is irregularly thickened by deposition of chromatin. x 6,300.

Fig. 9—Part of a cell 24 hours after inoculation with the D-43 strain. In the nucleus, clusters of viral particles and finely granular material formed a dense zone, which occupies the central area. This area corresponds apparently to the site of an intranuclear inclusion body in an adjacent thick section shown in the inset. A peripheral pale zone also contains numerous dispersed viral particles. x 7,800. Inset shows the same cell observed in an adjacent thick section stained by Giemsa's method. x 1,350.

Fig. 10—A portion of a cell 24 hours after inoculation with the D-43 strain. The central area of the nucleus appears to correspond to the site of an intranuclear inclusion body in an adjacent thick section shown in the inset and contains only a few clusters of viral particles. Numerous viral particles are accumulated in the peripheral portion of the nucleus. x 8,700. Inset shows the same cell seen in an adjacent thick section stained by Giemsa's method. x 1,350.





Figures 11 and 12 are electron photomicrographs of material from which no embedding plastic was removed.

Fig. 11—Part of a cell 36 hours after inoculation with the D-43 strain. A nucleus which contains dispersed viral particles, clumps of finely granular material, and a dense nucleolus is present in the center of the field. The cytoplasm also contains a small number of dispersed viral particles. Mitochondria have disintegrated and vacuolization is prominent. x 10,500.

Fig. 12—Forty-eight hours after inoculation with the Matsuda strain. A portion of a nucleus filled with numerous viral particles of varying density. In the upper right portion of the photomicrograph, viral particles embedded in the finely reticulated material are arranged in a crystalline array. The nuclear membrane is seen in the upper left corner. x 30,600.

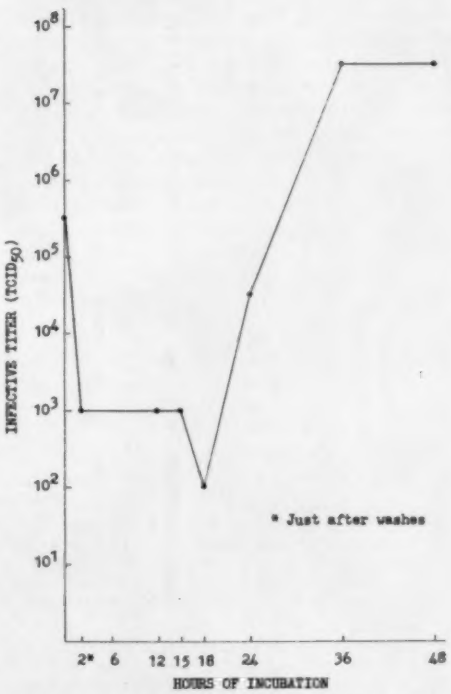
inclusion body because of severe nuclear pyknosis (fig. 4). When examined by the electron microscope, the nuclei of these cells generally contained no solid structures reminiscent of the intranuclear inclusion bodies seen with the light microscope. Viral particles, either singly or in clusters, and clumps of the finely granular material were scattered throughout the nucleus (fig. 11). Some nuclei, however, contained large compact aggregates of viral particles. In cultures inoculated with the Matsuda strain, the viral particles were frequently arranged in a crystalline array (fig. 12–14). Similar to findings in 24-hour specimens, occasionally only a few clusters of viral particles appeared to be embedded in the central mass, while numerous particles accumulated in the nuclear margin.

Intranuclear viral particles were round to oval and varied in density. Dense particles in which no internal structure was resolved had relatively indistinct margins. They were approximately 55 mμ in diameter as determined from the average of the longest and the shortest axis. Less dense particles were a little larger than the former, averaging about 64 mμ in diameter. Studied at high magnification, a thin, limiting membrane was often discernible in these particles. Three types could be distinguished in the less dense particles by internal structure: particles with homogeneous contents of low density, those with an internal body about 20 mμ in diameter, and those with a core of low density, giving the appearance of a central cavity (fig. 13–15).

In cells of cultures harvested 24 hours or more after inoculation, dispersed viral particles were frequently identified in the cytoplasm of those with severe nuclear changes (fig. 11, 16, 17). In such cells, defects in the nuclear membrane were repeatedly observed, and sometimes viral particles appeared to be escaping from these disruptions of the membrane into the cytoplasm (fig. 13). In these instances, viral particles were also found outside of cells, attaching to their external surface or lying free in the intercellular spaces (fig. 16). Cytoplasmic and extracellular particles were similar in shape and size to those observed in the nucleus, but were composed mostly of dense particles and less dense particles with an inner body. Cytoplasmic alterations characterized by swelling and disintegration of the cytoplasmic organelles, vacuolization, an increase in number of lipid inclusion bodies, and of irregular cytoplasmic projections increased with the lapse of time after infection. However, alterations of the same kind were also encountered in degenerated cells of uninoculated cultures. Laminated structures consisting of multiple membranes arranged in concentric layers were occasionally observed in the cytoplasm of cells at advanced stages of infection (fig. 17).

Virus Assay

Periodic titrations of virus in the culture fluid, performed in a number of experiments, gave uniform results. The infective titer, which had decreased after washing at the 2-hour point, began to increase at some time between 18 and 24 hours after inoculation and reached the maximum titer ($10^{6.5}$ to $10^{7.5}$ t.c.i.d.$_{50}$ per ml.) after 36 hours (graph 1).



Graph 1—Growth curve of the D-43 strain of infectious canine hepatitis virus in cultures of dog kidney cells.

DISCUSSION

It is apparent from the observations that the intranuclear inclusion bodies associated with ICH virus infection are formed in the course of virus multiplication. In thin sections of cells infected with herpes simplex virus,[2,21] herpes B virus,[27] and equine abortion virus,[4] respectively, it was impossible to visualize with the electron microscope an intranuclear structure resembling the type A inclusion body as seen with the light microscope. In the case of ICH virus herein reported, there was, as a rule, a good correlation between the morphologic appearance of the nuclear alterations observed by the electron microscope and by the light microscope. When studied by the electron microscope, most of the inclusion bodies appeared to consist of dense, finely granular material and viral particles. Their fine structure is consistent, in some degree, with that described by Luse and Smith [19] in the case of salivary gland virus and that described by Faweett [10] in the case of the Lucké renal adenocarcinoma. However, the inclusion bodies appeared to be not always associated with the presence of viral particles, and the inclusion bodies, especially those in advanced stages of development, were not necessarily identifiable in thin sections, using the electron microscope.

It is interesting to correlate the fine structures of the inclusion bodies with the stages of their development. The earliest inclusion bodies consisted of several eosinophilic granules. In electron micrographs, the majority of them appeared free from viral particles and consisted of dense, finely granular material which accumulated at multiple foci in the nucleus. Shortly after this stage, viral particles made their appearance in close apposition with the granular material. Then discrete clumps of granular material and the viral particles appeared to coalesce in the central area of the nucleus, forming a single central mass which was visible as a classical intranuclear inclusion body, using the light microscope. The sequence of development of the inclusions just described seemed to be similar to that disclosed by Goodpasture [13] from his histologic studies on animals infected with herpes virus and by Fujimoto,[12] reporting on animals infected with ICH virus. Occasional cases of infection suggest, however, that the viral development takes place not only in the inclusion bodies but anywhere within the nucleus, particularly in the inner zone of the nuclear membrane where finely granular material accumulates. With the advance of infection, the granular material gradually decreased in amount, while the viral particles increased in number and finally were dispersed all over the nucleus. At this stage, the inclusion bodies were no longer visible as a solid structure. Thus, whether or not the inclusion bodies are visible in thin sections under electron mieroscopy appears to depend, to a great extent, on the amount of the osmiophilic granular material and the degree of aggregation of viral particles. Within the nucleus, the finely granular material accumulated prior to the development of the viral particles and decreased gradually in amount with the increase in number of the viral

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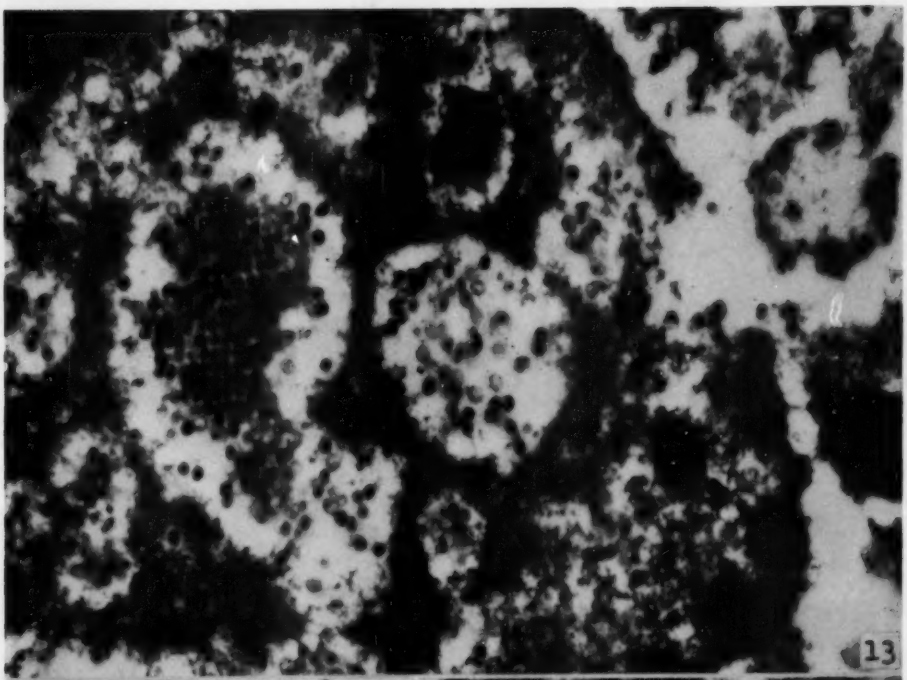
Figures 13 to 15 are electron photomicrographs of material from which no embedding plastic was removed.

Fig. 13—A portion of a nucleus 48 hours after inoculation with the Matsuda strain. Scattered viral particles and crystals lie in networks of reticulated condensation of the dense granular material. Four types can be distinguished among these particles. The nuclear membrane traversing the right side of the field has been disrupted, and viral particles appear to be in the process of being released from the nucleus into the cytoplasm through a break of the membrane. x 37,600.

Fig. 14—Forty-eight hours after inoculation with the Matsuda strain. Part of a nucleus showing a cluster of viral particles in crystal-like arrangement at higher magnification. Most of the particles are uniformly dense and have no internal structure. x 66,000.

Fig. 15—Portion of the nucleus of a cell 48 hours after inoculation with the Matsuda strain. Various types are discernible among viral particles. The nuclear membrane is in the upper left corner. x 51,000.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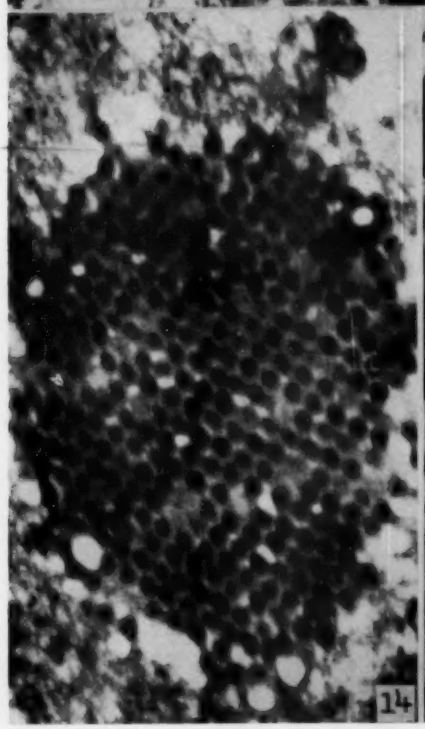
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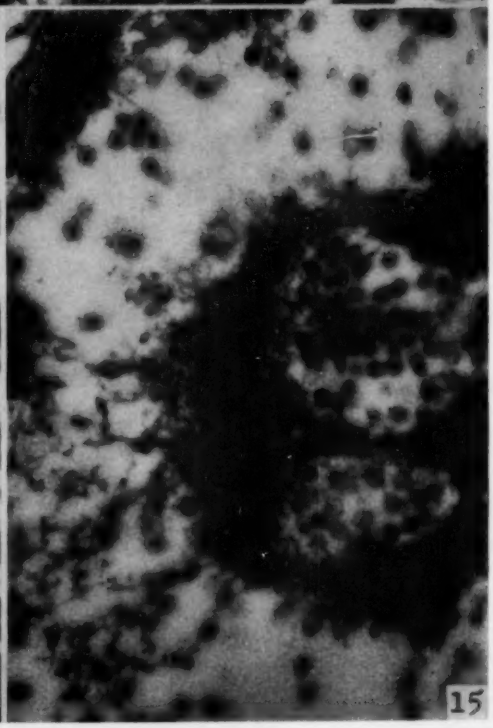
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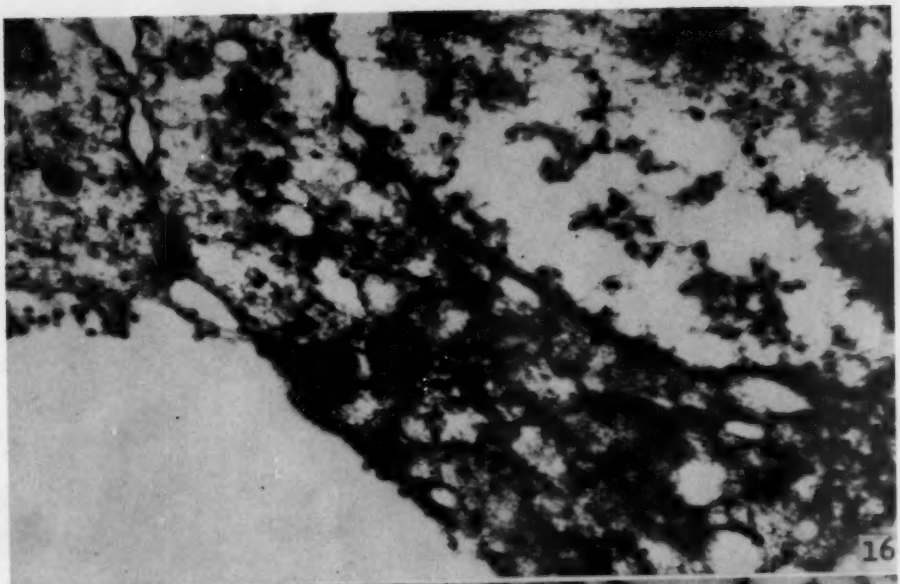

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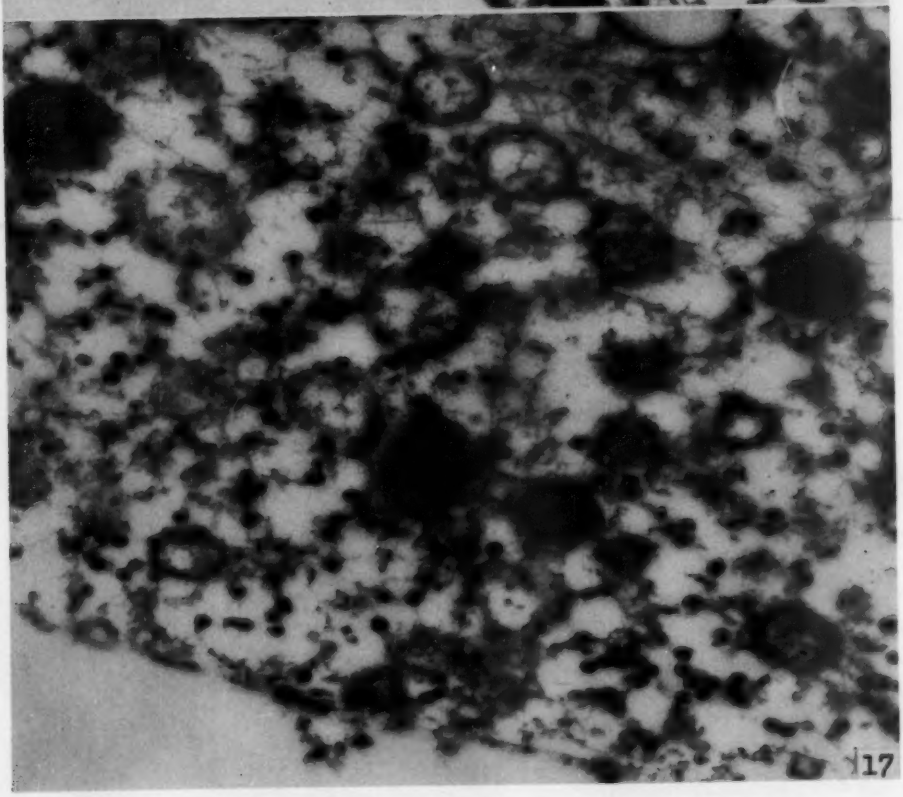

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particles. These findings suggest that this material may be utilized for viral particle formation.

Crystalline aggregates of viral particles as illustrated in this report have often been observed in sections of cells infected with some other virus [3, 5, 14-16, 18, 22-25, 28] and in purified preparations of viruses.[1, 20, 29, 32] The fact that the Matsuda strain of ICH virus is liable to form crystalline aggregates of viral particles in the infected nucleus was first reported by Tokui *et al.*[31] This was confirmed by the present authors. In contrast, the D-43 strain of the same virus, cultivated under similar conditions, has never shown any regular arrangement of viral particles. So far as the strains of ICH virus are concerned, whether or not viral particles crystallize appears to depend greatly upon the property of the strain observed.

The viral particles originally produced in the nucleus appeared to be released into the cytoplasm when the nuclear membrane was disrupted and, ultimately, into the culture medium when cytolysis occurred in the host cell. The fact that a rise in virus titer in the culture medium took place a little later than the appearance of viral particles within the nucleus might be explained from these observations.

The particle size measured in the present work was slightly smaller than that previously reported.[30] This difference might be explained by the dissimilarity of the method of measurement. The viral particles were classified morphologically into 4 types. Their morphologic differences appear to be explained by the presumption made by Morgan *et al.*[22] for viruses of the RI-APC group; *i.e.*, the differences are mostly related to the function of the viral particles. In this connection, the fact that cytoplasmic and extracellular particles were almost exclusively composed of dense particles and less dense ones with a central body may suggest that these 2 types of particles are at more advanced stages of development than the others.

No cytoplasmic alterations related directly to viral infection were detected. The laminated structures observed in the cytoplasm of cells at advanced stages of infection resembled those described by Donald and Liu [9] in chicken embryo cells infected with the virus of primary atypical pneumonia, and by De Robertis and Sabatini [8] in the adrenal cortex of normal hamsters. The origin and significance of such structures, however, still remain to be determined.

SUMMARY

Monolayer cultures of canine kidney cells were infected with infectious canine hepatitis virus. The morphologic changes occurring in these cells were sequentially studied with light and electron microscopes, together with tests of the amount of infective virus produced.

Intranuclear inclusion bodies, which consisted mostly of several eosinophilic granules under the light microscope and which were seen as multiple condensations of dense, finely granular material under the electron microscope, appeared 18 hours following inoculation and shortly before the appearance of infective virus in the culture fluids. Then viral particles developed in close apposition with the granular material within the nucleus and increased in number parallel to a rise in virus titer in the culture fluids. Discrete clumps of the granular material and clusters of viral particles coalesced gradually near the

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Figures 16 and 17 are electron photomicrographs of material from which no embedding plastic was removed.

Fig. 16—Portions of 2 cells from a culture 36 hours after inoculation with the D-43 strain. Viral particles can be seen in the nucleus and the cytoplasm of a cell occupying the right two thirds of the field. They are also identifiable on the external surface of both cells. x 25,500.

Fig. 17—An area of the cytoplasm of a cell 48 hours after inoculation with the D-43 strain. Viral particles, among which dense bodies with no apparent internal structure predominate, are scattered throughout the field. Mitochondria appear to be in various stages of disintegration. Several multilaminated structures can be seen. x 38,400.

center of the nucleus, forming a single, large, central mass that was reminiscent of a classical inclusion body seen under the light microscope at about 24 hours. In later stages of infection, the granular material in the central mass decreased in amount and dispersed so widely with the viral particles throughout the nucleus that it became difficult to observe any clearly outlined structures in thin sections. In some instances, however, viral particles apparently were not contained in the central mass, but rather appeared to develop in the granular material of the nuclear periphery.

The viral particles could be divided into 4 types: dense and solid bodies, less dense bodies characterized by a thin membrane and homogeneous content, those having a dense central body, and those with a transparent core. They were round to oval in shape, and their diameter was approximately 55 mμ in the dense bodies and 64 mμ in the less dense bodies, regardless of their type. Frequently, they formed intranuclear crystalline aggregates in cells infected with the Matsuda strain of virus, but not in cells infected with the D-43 strain. It was suggested that the finely granular material might be utilized for viral particle formation. The viral particles were released into the cytoplasm through breaks of the nuclear membrane and eventually outside the cell by cytolysis. The cytoplasmic alterations observed in the infected cells could not be considered as specific for viral infection.

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SUMMARIO IN INTERLINGUA

Microscopia Electronic de Virus de Infectiose Hepatitis Canin, Mantenite in Culturas de Canin Cellulas Renal

Culturas monostratal de canin cellulas renal esseva inficite con virus de infectiose hepatitis canin. Le alterationes morphologic occurrente in iste cellulas esseva studiate sequentialmente per microscopia a lumine e electronic. Esseva etiam testate le quantitate de virus infective producite.

Corpores de inclusion intranuclear—que consisteva principalmente de plure granulos eosinophile in le microscopio a lumine e que esseva vidite como multiple condensationes de dense e finmente granular material sub le microscopio electronic—appareva 18 horas post le inoculation e brevemente ante le apparition del virus infective in le liquidos cultural. Postea particulas virusal se disveloppava in proximissime apposition al material granular intra le nucleo e cresceva in numeros in parallela con le augmento del titro virusal in le liquidos cultural. Discrete cumulos del material granular e cumulos de particulas virusal coalesceva gradualmente in le vicinitate del nucleo pro formar un sol e grande massa central que rememorava le classic corpore de inclusion que es vidite sub le microscopio a lumine post circa 24 horas. In stadios subsequente del infection, le material granular in le massa central declinava in quantitate e se dispergeva con le particulas virusal si extensemente in omne partes del nucleo que il deveniva difficile observar ulle clarmente delineate structuras in tenue sectiones. In certe casos, del altere latere, particulas virusal esseva apparentemente non presente in le massa central sed pareva disveloppar se in le material granular del peripheria del nucleo.

Le particulas virusal poteva esser dividite in quatro typos: Dense e solide corpores; minus dense corpores, characterisate per un tenue membrana e un contento homogenee; particulas con un dense corpore central; e particulas con un centro transparente. Illos esseva ronde o oval in conformation. Le diametro esseva approximativemente 55 mμ in le dense corpores e 64 mμ in le minus dense corpores, sin reguardo al typo. Frequentemente illos formava aggregatos crystallin intranuclear in cellulas inficite con le racia Matsuda del virus sed non in cellulas inficite con le racia D-43. Esseva facite le suggestion que le material finmente granular es forsan utilisate in le formation de particulas virusal. Le particulas virusal esseva liberate a in le cytoplasma per rupturas del membrana nuclear e, plus tarde, per cytolyse al exterior del cellulas. Le alterationes cytoplasmic observate in le inficite cellulas non poteva esser considerate como specific pro infection virusal.

Electron Microscopy of Equine Abortion Virus

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SEQUENTIAL NUCLEAR alterations occurring in hepatic cells of hamsters infected with equine abortion virus (EAV) were thoroughly studied with the light microscope by Randall and Bracken.[14] They observed progressive accumulation of Feulgen-positive material in infected nuclei and also noticed a definite relationship existing between the nuclear changes and the appearance of progeny virus both in the liver and in the blood of infected animals. These observations seem to suggest that the intranuclear inclusion bodies characteristic of this viral infection might be associated in some way with viral multiplication. Recently, Bracken and Norris[1] employed the electron microscope for their investigation and identified viral particles in the nuclei of the hepatic cells of experimentally infected hamsters. They failed, however, to elucidate the relationship of the intranuclear inclusion body to the viral particles or to the site of viral replication, since no structures identifiable as inclusion bodies had been seen.

It seems worth while to study the virus grown in a different type of host cell. Shimizu *et al.*[18] reported that EAV propagated with readily recognizable cytopathic effect in horse kidney cells in tissue culture. Such a host cell system has a number of advantages for studying a virus-host cell relationship, as has been described by Reissig and Melnick[15] in their study with herpes B virus.

It was the purpose of this study to observe sequentially, with the light and the electron microscopes, morphologic changes occurring in cultured horse kidney cells following infection and to relate these findings to the appearance of virus within the cells and in the culture fluid as determined by infectivity titrations. In addition, the hepatic cells of hamsters infected with the same virus were also examined morphologically.

MATERIALS AND METHODS

Virus.—The hamster-adapted Kentucky D strain of EAV, supplied by Dr. E. R. Doll, Kentucky Agricultural Experiment Station, Lexington, was maintained by serial passages in horse kidney cell culture and was used at the 30th passage level. The infective titer was 1.7×10^7 plaque-forming units (p.f.u.) per milliliter when determined by the plaque count method on horse kidney cell culture. In some experiments, the H 45 strain, isolated by Shimizu *et al.*[17] in equine kidney cell culture from a horse in Japan which aborted naturally, was also used.

Tissue Culture.—Monolayer cultures of kidney cells from a young adult horse were prepared by the trypsinization method described by Youngner.[24] The growth medium employed was Hanks' solution containing 0.5 per cent lactalbumin hydrolysate and 5.0 per cent bovine serum. To each milliliter of the medium were added 100 units of penicillin and 100 μg. of streptomycin. The primary cultures were prepared in Petri dishes, 100 mm. in diameter, and incubated in an atmosphere containing 5.0 per cent CO_2 in the air. When a satisfactory monolayer of cells was formed, usually within a week at 37 C., the cultures were washed once with 10 ml. of phosphate-buffered saline solution (PBS) and treated with 5 ml. of 0.25 per cent trypsin in PBS. The resulting cell suspension was washed with the nutrient medium by centrifugation and resuspension, and then counted for the number of cells in a hemocytometer. The secondary cultures, prepared in Petri dishes (50 mm. in diameter), were used for the present work. For the morphologic examination, several cover slips, 6 by 18 mm., were placed in a dish before seeding with the passage cells. Each culture was started with 5 ml. of a suspension containing about 100,000 cells.

Infection of Culture.—After incubation for 2 to 3 days at 37 C., a monolayer of cells grew on the glass surface of the Petri dish as well as on the cover slips. Each culture was washed with 5 ml. of PBS and inoculated with 1 ml. of the virus in a nutrient medium containing 1.7×10^7 p.f.u. The dose of virus had been calculated so that 10 p.f.u. were available per cell. After 3 hours' incubation

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at 37 C., the cultures were washed 4 times with 5 ml. of cold PBS. Five milliliters of fresh nutrient medium containing 5 per cent bovine serum was then added to each culture, which was reincubated at 37 C.

Sampling and Titration.—Infected cultures were harvested at various intervals after infection, as will be described. Three cultures were removed at each selected interval, 1 containing cover slips for morphologic observation and the remaining 2, without cover slips, for titration of virus. For the titration of virus, the fluids were harvested from each 2 cultures, pooled, and centrifuged at 2,000 r.p.m. for 10 minutes to remove cells or cell debris. The supernatant fluid, titrated by the plaque method, was regarded as the extracellular virus. The cells remaining in the Petri dishes were gently washed twice with 5 ml. of cold PBS, scraped with a rubber policeman, and suspended in 5 ml. of nutrient medium. The cell suspension contained in the tube was rapidly frozen in a dry-ice alcohol bath and then rapidly thawed at 37 C., the same procedure being repeated twice. The disrupted cell suspension was centrifuged at 2,000 r.p.m. for 10 minutes, and the supernatant fluid was tested for virus by the plaque technique and considered as cell-associated virus.

The titration by the plaque technique was performed as follows: The monolayer culture in a Petri dish (50 mm. in diameter) was washed once with 5 ml. of PBS, and 0.2 ml. of virus dilution in nutrient medium was inoculated. The virus was adsorbed at 37 C. for 3 hours, and then 5 ml. of nutrient agar was overlaid. The agar overlay was composed of Earle's saline solution, containing 1 per cent Bacto-agar (Difco), 0.5 per cent lactalbumin hydrolysate, 0.1 per cent yeast extract, and 10 per cent bovine serum. The dishes were incubated at 37 C. in an atmosphere of 5 per cent CO_2 in air. Plaque counts were made on the fourth day after inoculation by staining with 5 ml. of 1:5,000 neutral red in PBS for 4 hours. Usually, 2 dishes were used for any given dilution of virus tested.

Preparation of Specimens for Microscopy.—The methods used to prepare specimens for electron and light microscopy have been previously described.[21]

An Additional Experiment with Hamsters.—Syrian hamsters, weighing about 40 Gm., were inoculated intraperitoneally with 1 ml. of a 10 per cent saline suspension of hamster liver infected with the Kentucky D strain of EAV. Infected animals were killed *in extremis* 17 and 28 hours after inoculation.

For an electron microscopic study, small blocks of the liver (harvested immediately) were fixed in osmium tetroxide, dehydrated in alcohols, embedded in methacrylate, and sectioned according to methods described elsewhere.[20]

For a comparative light microscopic study, thick sections were cut from the same tissue blocks as were used for electron microscopy. They were mounted on slides and stained with the hematoxylin and eosin variant according to the technique recommended by Jennings *et al.*[5] In addition, some other blocks were harvested from the same liver as that from which the specimens for electron microscopy had been collected, fixed in Zenker's acetic acid fluid, embedded in paraffin, cut at 5 μ, and stained with hematoxylin and eosin.

RESULTS

EQUINE ABORTION VIRUS IN CULTURED HORSE KIDNEY CELLS

Light Microscopy.—Cells from infected cultures were found to have no pathologic changes until 3 hours following inoculation. At that time, a few cells in preparations stained with hematoxylin and eosin had undergone a nuclear alteration considered to be the first manifestation of infection. In the nuclei of these cells, the fine, evenly distributed, chromatin network appeared to be replaced by fine granules and, in some areas, to be condensed into irregular strands or coarse reticulated masses. Four hours after infection, cells with these nuclear changes increased in number. In some, the nucleus contained 1 or more small, light-pink-staining, irregular-shaped bodies, presumably inclusion material, in the interstices of the strands of condensed chromatin (fig. 1). At this stage of infection, the nucleoli, usually 2 or more in a nucleus, were relatively well preserved but sometimes were displaced to the peripheral portion of the nucleus. No cytoplasmic changes could be detected, even in the cells with these altered nuclei. Giemsa's method was found to be inadequate in demonstrating these early nuclear alterations.

A majority of the cells in cultures 6 hours after inoculation contained evidence of infection. The inclusion material was accumulated in the nuclei of almost half of the cells examined. Single, large, inclusion bodies, stained faintly with eosin, were also observed in scattered cells. They were usually surrounded by a faint halo, and appeared to change shape according to the contour of the chromatin-free area in which they were contained. This type of inclusion was considered to be formed by the coalescence of small, multiple, inclusion material. From 8 to 10 hours after inoculation, there was an increase in the number of nuclei containing inclusions (fig. 3). Some nuclei were filled with a homogeneous mass staining bluish-pink and resembling the full inclusion body described by Scott *et al.*[16] in rabbit corneal cells infected with the virus of herpes sim-

plex. At 8 hours, another type of cellular alteration characteristic of the later stages of infection began to be observed. Scattered throughout the culture were cells having a retraction of both the cytoplasm and the nucleus, with increasing basophilia. They remained attached to their neighbors with thin cytoplasmic strands (fig. 2). It frequently became difficult to recognize the inclusion in these cells because of dark staining of their nuclei, but if the cells were carefully stained and differentiated, their inclusions (resembling either the shrunken type A or full variety) could be seen in most cases. The nucleoli of such nuclei were displaced to the peripheral portion and often adhered to the nuclear membrane.

During the 12 to 36 hours following infection, the cells with the described changes increased in number until the culture was almost completely destroyed. In the majority of cells, there was marked loss of cytoplasmic processes and a further increase in both cytoplasmic and nuclear basophilia. Cells were gradually detached from the surface of the glass, gaps being left in cell sheets.

Electron Microscopy.—Normal cells from monolayer cultures of horse kidney cells observed in the present work had a structure basically similar to that described in monkey and dog kidney cells in tissue cultures.[15, 21]

During the first 4 hours following infection, the great majority of cells were morphologically indistinguishable from uninoculated control cells. Only occasional cells in sections prepared at the 3- and 4-hour stages of infection revealed the first noticeable nuclear changes, believed to be related to viral infection. In the nuclei of these cells, the chromatin decreased in amount in the central zone or was aggregated into irregular-shaped masses of varying size with indefinite outline, leaving clear areas between them (fig. 6). The chromatin was also somewhat marginated, and the nucleoli were usually displaced to the peripheral portion of the nucleus. Although such alterations of nuclei appeared to be comparable, in some degree, to early nuclear changes seen in the histologic preparations, neither such inclusion material as observed under the light microscope nor structures presumed to be viral particles could be demonstrated in them. However, it was difficult in these early stages to determine whether viral particles were actually lacking or were present in such small

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Figures 1 to 5 are light photomicrographs. Figures 1 to 3 were taken from monolayer cultures of horse kidney cells grown on cover slips; and figures 4 and 5 were taken from sections of hamster liver infected with Kentucky D strain of equine abortion virus.

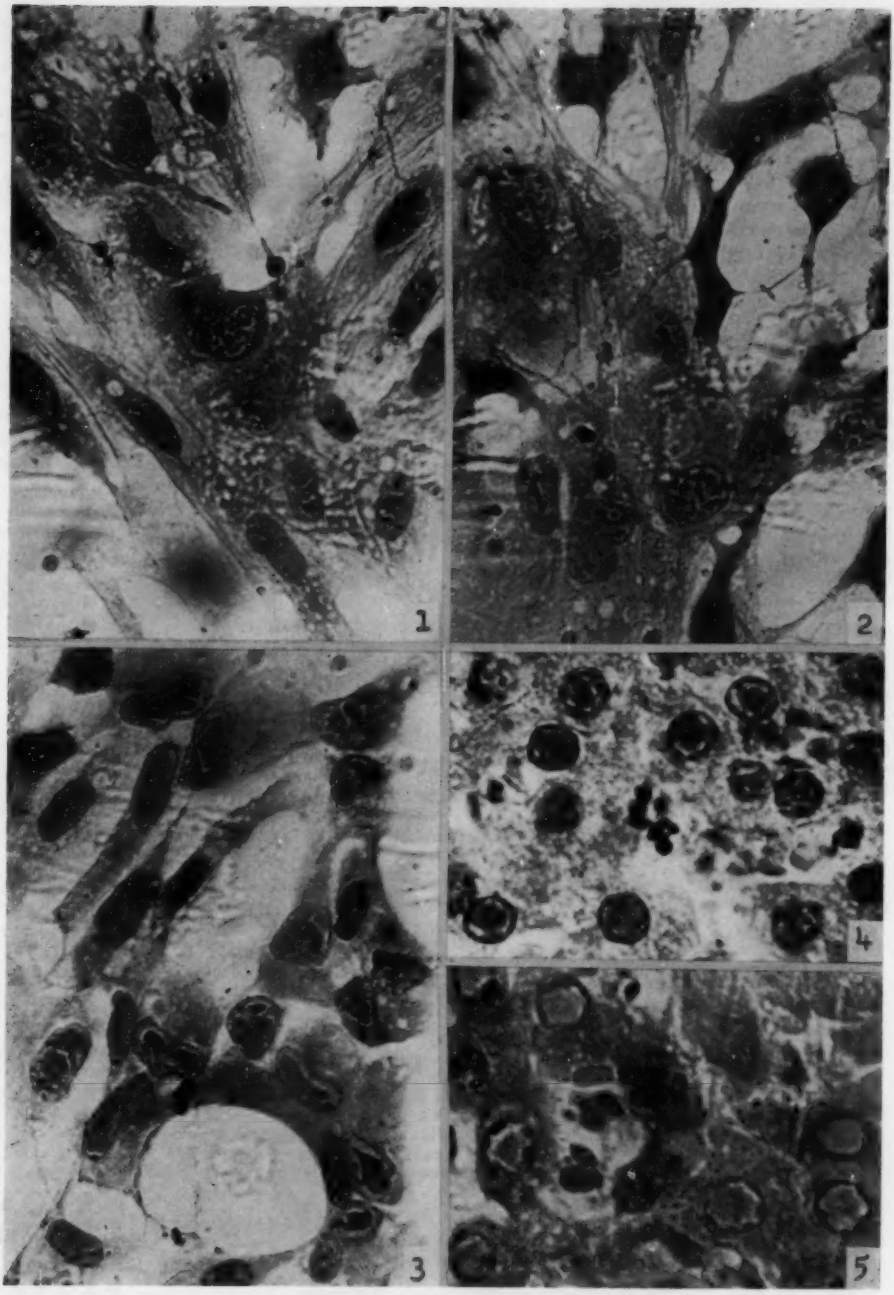
Fig. 1—Cultured cells harvested 4 hours after inoculation, fixed in Bouin's fluid, and stained with hematoxylin and eosin. In the nuclei of 2 cells near the center of the picture, multiple accumulation of acidophilic inclusion material can be seen. A few of the other cells also have early nuclear change, but do not contain inclusion material. x 600.

Fig. 2—Cells from a similar culture harvested 8 hours after infection, fixed and stained with the same procedure as that for figure 1. Acidophilic intranuclear inclusions, single large, or multiple small, are seen in many cells. In several cells there is retraction of both the cytoplasm and the nucleus, and increased basophilia. x 600.

Fig. 3—Cells from a culture 10 hours after infection. The nuclei of most cells contain a single, large, irregular-shaped inclusion. The cells were fixed in absolute methanol and stained with Giemsa's solution. x 600.

Fig. 4—A section of the liver of a hamster killed in extremis. The tissue was fixed in Zenker's acetic acid fluid, embedded in paraffin, cut at 5μ, and stained with hematoxylin and eosin. Practically every cell contains a classical type A inclusion. Margination of chromatin is conspicuous. x 1,350.

Fig. 5—A thick section cut from the same liver block as that from which an electron micrograph shown in figure 16 was taken, and stained with hematoxylin and eosin. The intranuclear inclusions seen in the hepatic parenchymal cells have been stained homogeneously with eosin and are separated from the nuclear membrane by a clear narrow zone. x 1,350.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numbers as to escape detection by electron microscopy.

In 6- to 10-hour sections, cells with the described nuclear changes were more frequently encountered and some of them had nuclei containing particles with a distinctive appearance not seen in the uninfected control cells (fig. 7-9). These particles, thought to represent viral forms, were present either individually in the clear areas between the aggregates of chromatin (fig. 7) or in clusters in the proximity of the nuclear membrane (fig. 8). They were apparently increasing in number as time lapsed. They were round or ovoid in shape and averaged approximately 95 mμ in diameter. They were variable in structure; some consisted of a dense membranous shell of varying thickness and a clear or homogeneous central zone of low density; others contained an electron-dense inner body, 25 to 45 mμ in diameter, eccentrically located within the clear central zone; and still others were uniformly electron-dense with no evidense of internal structure (fig. 9). Occasional particles, especially those consisting of a thin limiting membrane and homogeneous contents, had incomplete membranes (fig. 9), but it was not clear whether these discontinuities of the limiting membranes were an artifact sustained during preparation of the specimen or were a real phenomenon representing a stage of their development.

Most of the cells harvested from 12 to 20 hours after inoculation contained viral particles in their nuclei, and the number of particles per nucleus increased with time. Although nuclear alterations varied from cell to cell, the nuclear chromatin was, as a rule, more closely aggregated and marginated, together with the nucleoli, to the periphery of the nucleus. Thus, the central clear zone of the nucleus in which viral particles were randomly distributed increased in size and eventually occupied most of the nucleoplasm. In such instances, the nucleus sometimes looked like a sac containing scattered viral particles, and only a small amount of chromatin was visible just inside the nuclear membrane (fig. 10). Through the course of infection, most cells had no structures identifiable as intranuclear inclusion bodies. The region occupied by an inclusion body appeared to correspond to the central clear zone of the nucleus. However, some nuclei, which seemed to be at advanced stages of degeneration and to correspond to pyknotic nuclei as observed in the histologic preparations, contained a large, central, reticulated mass with a surrounding clear zone. Such structures were, in shape and location, reminiscent of a shrunken classical intranuclear inclusion body seen with the light microscope but contained few or no viral particles (fig. 11).

Cells harvested in the later stages of infection, such as 24 and 36 hours after inoculation, generally had either nuclei with dispersed viral particles (fig. 12) or retracted nuclei with a large, central reticulated mass as illustrated (fig. 11). However, even at these progressed stages of infection, there were occasional cells with the nuclear changes common in the earlier stages.

Cells infected 6 hours earlier had no lesion of the cytoplasm that could be distinguished from that of uninfected con-

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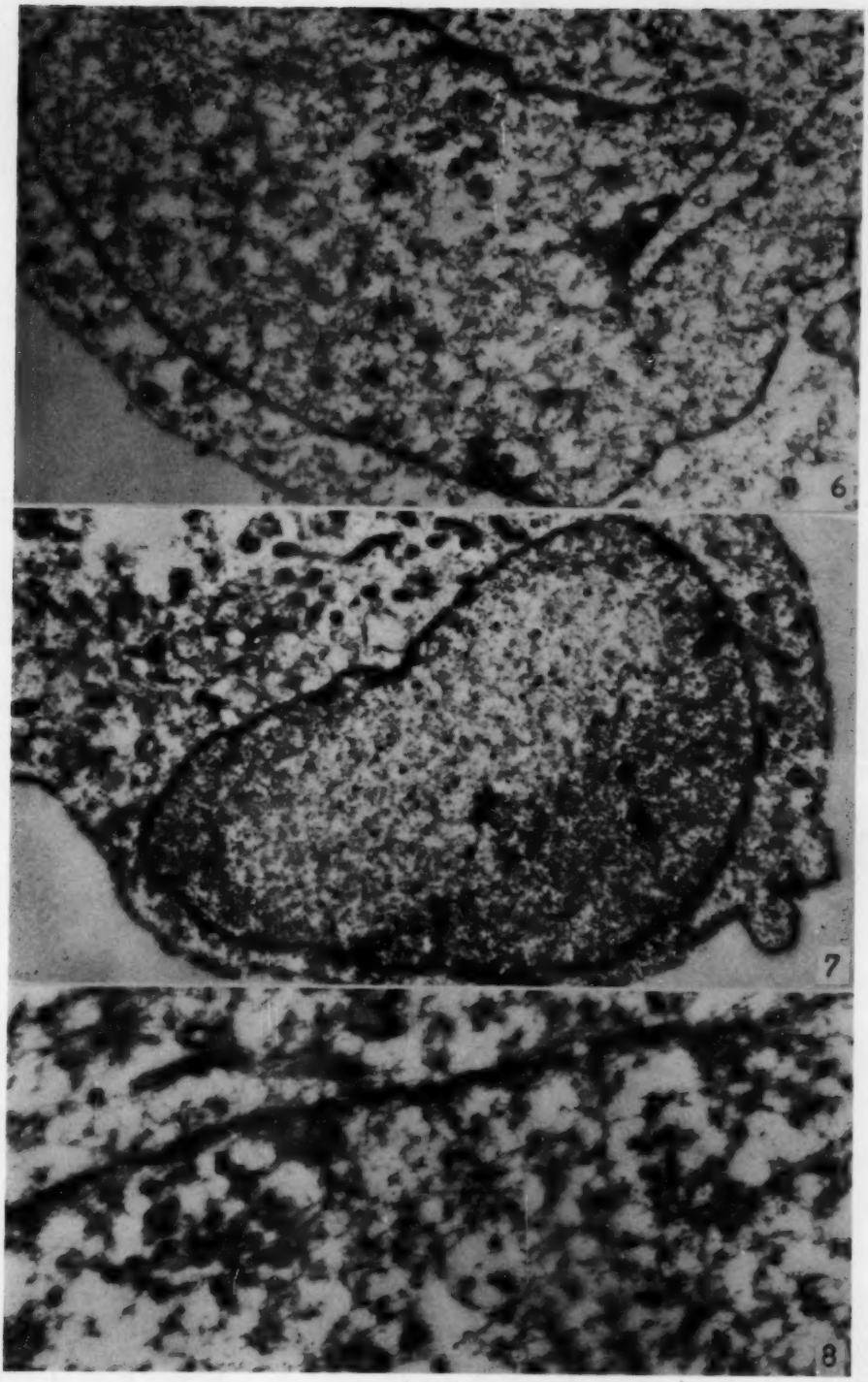
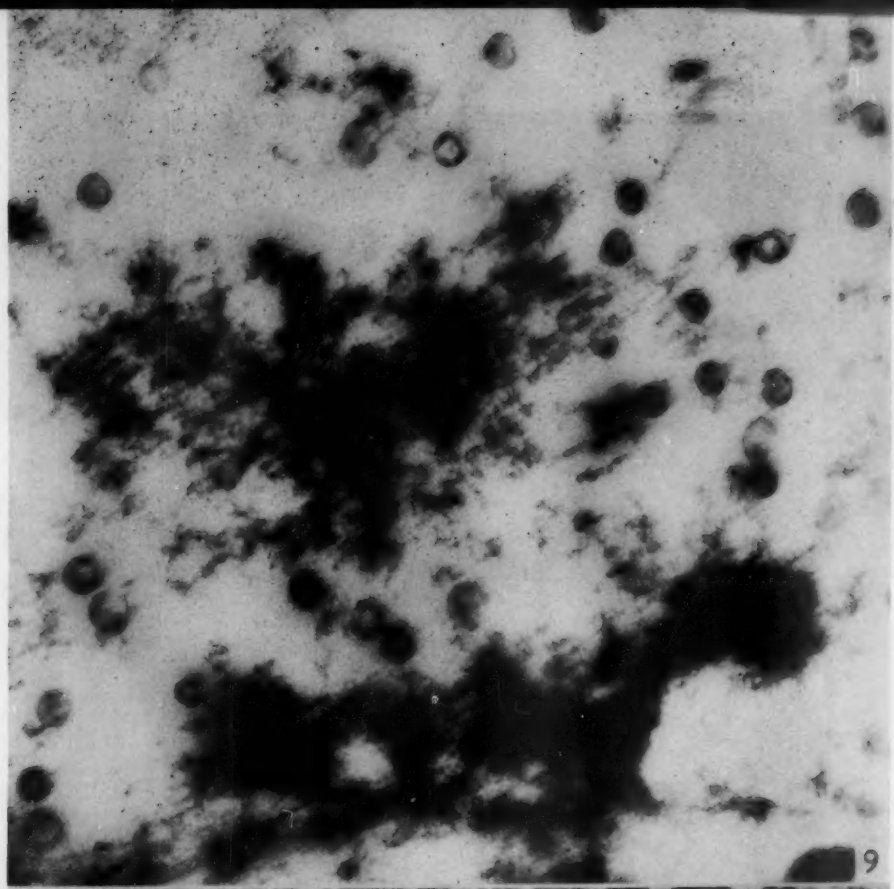
Figures 6 to 15 are electron micrographs taken of thin sections prepared from cultured horse kidney cells infected with the Kentucky D strain of equine abortion virus.

Fig. 6—Portion of a cell 4 hours after inoculation. In the nucleus which occupies most of the picture, chromatin is irregularly clumped and somewhat marginated. The nucleolus is not shown. Particles presumed to be virus have not yet appeared. x 14,700.

Fig. 7—A cell harvested 6 hours after inoculation. A clear area, in which viral particles are scattered, makes its appearance near the center of the nucleus. The chromatin is marginated and the nuclear margin appears irregularly thickened. The nucleolus is not included in this level of section. x 10,200.

Fig. 8—Part of a cell 6 hours after inoculation. The nuclear membrane is seen across the upper portion of the micrograph and the cytoplasm free of virus is above. Two small clumps of viral particles can be seen just inside the nuclear membrane. All the particles are less dense and some contain a dense inner body. x 28,800.





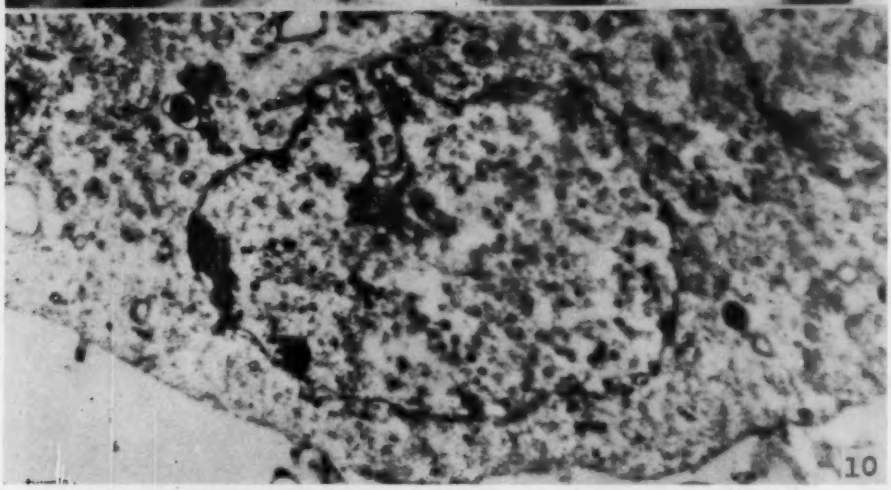


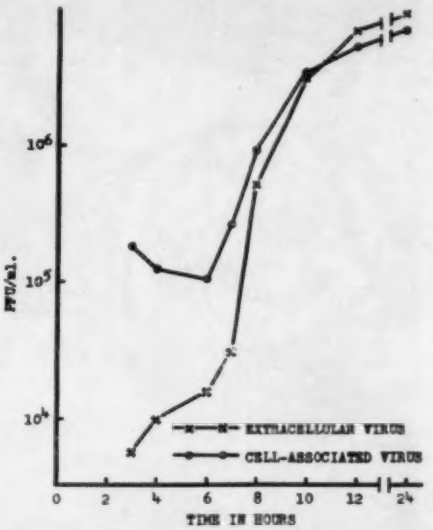
Fig. 9—Portion of the nucleus of a cell 10 hours after inoculation. Dispersed viral particles are shown at high magnification, various types being distinguished among them. In some of the less dense particles, the limiting membrane appears to have been disrupted. The nuclear membrane, which is poorly defined in this section, traverses near the lower border of the picture horizontally. x 60,000.

Fig. 10—A cell harvested 12 hours after inoculation. Numerous dispersed viral particles are present in the nucleus which has lost most of its chromatin. The chromatin is in the form of small dense clumps adhering to the nuclear membrane. x 14,800.

trols. At 8 hours, some of those cells with nuclear changes had varying degrees of cytoplasmic alterations simultaneously. These were characterized by vacuolization, swelling and disruption of mitochondria, disintegration of mitochondrial cristae, and fragmentation of endoplasmic reticulum. At this stage, well-defined particles, presumed to be those of EAV, were first clearly seen in the altered cytoplasm. Thereafter, the number of involved cells and cytoplasmic particles per cell increased progressively with the lapse of time (fig. 13). As the degenerative process progressed, the cytoplasm of the cells was apparently retracted and filled with numerous, irregular-outlined vacuoles, giving the appearance of coarse reticulation (fig. 11, 12). The cytoplasmic particles were spherical or ellipsoidal in shape and measured approximately 106 x 121 mμ on the average. Most of them were uniformly dense, and no internal structure was resolved. However, some of them had a central region of diminished opacity, and still some others had a central region more dense than the remaining portion of the particle (fig. 14). The majority of the particles were lodged, either singly or in a cluster, within a cytoplasmic vacuole (fig. 11, 13, and 14), although some particles, especially those in cells at advanced stages of disintegration, were free in the cytoplasm. The cytoplasmic vacuoles containing particles varied in shape and size and were set off from the surrounding cytoplasm by a dense membrane with a single or double structure (fig. 14). In cultures harvested 8 hours or more after inoculation, particles which were identical in size and configuration with those seen in the cytoplasm were also found in the extracellular space (fig. 15).

Virus Assay.—Several experiments were performed by the technique outlined. The number of plaque-forming units was determined at specified time intervals after inoculation. Growth curves thus obtained were basically similar; an example is shown (graph 1).

In the case of cell-associated virus, the infective titer, after remaining almost unchanged during the first 6 hours, was found to increase some time between 6 and 7 hours after inoculation. This increase was followed by exponential reproduction for 5 hours and then the rate of increase diminished. The extracellular virus gradually increased during the first 7 hours.



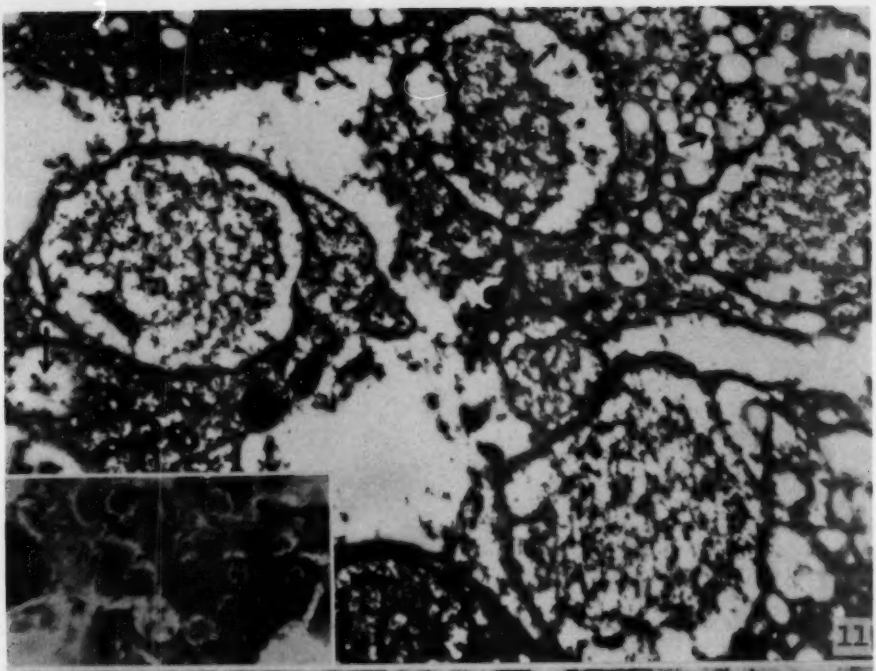
Graph 1—Growth curve of the tissue-culture adapted Kentucky D strain of equine abortion virus in cultures of horse kidney cells. Time 0 corresponds to the moment when virus was added to the cultures. At the 3-hour point, the cultures were washed 4 times with cold phosphate-buffered saline solution to remove most of the unadsorbed inoculum.

However, this was considered to be due to the gradual elution of inoculated virus which had remained adsorbed on the surface of cells through the process of repeated washing. The release of reproduced virus, which was indicated by a rapid increase in the virus titer of fluid phase, was manifested after about 7 hours.

EQUINE ABORTION VIRUS IN HEPATIC PARENCHYMAL CELLS OF HAMSTERS

In the foregoing experiment, it was generally impossible to visualize with the electron microscope an intranuclear structure resembling the inclusion body as seen in the light microscope. The liver of a hamster infected with EAV seemed to provide an excellent approach for studying the matter of inclusion bodies, since practically all the hepatic parenchymal cells contained the classical type A intranuclear inclusion as illustrated (fig. 4).

In electron micrographs, intranuclear viral particles, similar in size and appearance to those observed in the infected cultured cells, were readily identified in the



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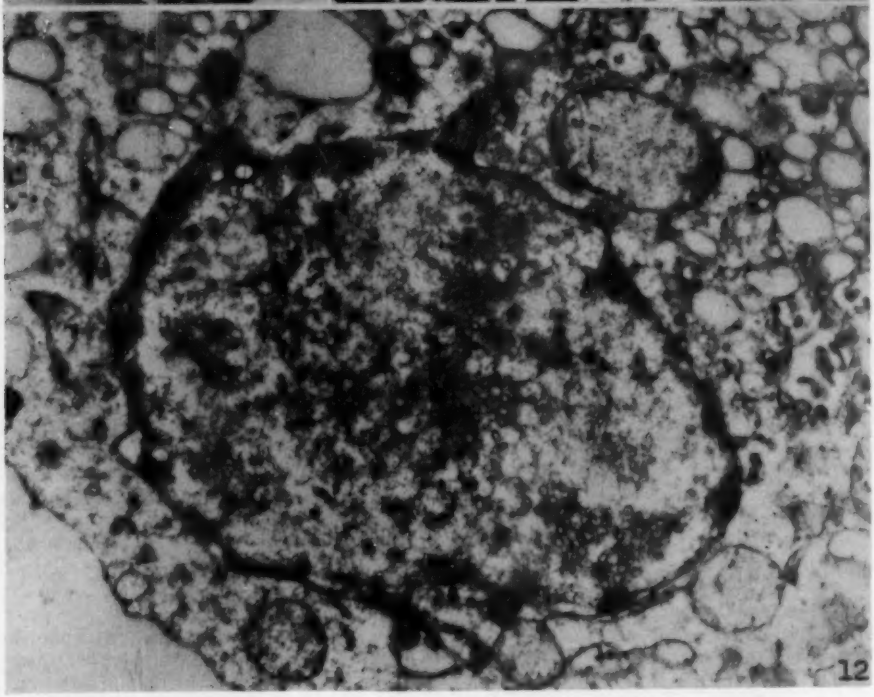

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nuclei of the great majority of the hepatic parenchymal cells (fig. 16–18). Cytoplasmic and extracellular particles were also observed. In infected cells, margination of chromatin and displacement of nucleoli to the periphery of the nuclei were seen clearly, whereas no solid, well-defined structures comparable with intranuclear inclusions were demonstrated in any of the cells examined (fig. 16). As observed in the cultured cells, the area apparently corresponding to the site of an inclusion body appeared rather clear. In these clear areas were observed a small amount of osmiophilic amorphous material and dispersed viral particles (fig. 17, 18).

Reissig and Melnick [15] have shown in monkey renal epithelial cells infected with herpes B virus that, when the cells were fixed in Zenker's fluid and stained with hematoxylin and eosin, typical inclusions were readily seen in the nuclei; in the cells fixed in buffered osmium tetroxide and stained similarly, no clear-outlined intranuclear inclusion bodies were found. In the present work, however, clear-outlined inclusion bodies with faint halo were observed in the nuclei of the cells fixed either in Zenker's acetic acid fluid or in buffered osmium tetroxide, though in the latter case inclusion bodies were more homogeneously stained with eosin and much less retracted than in the former (fig. 5).

DISCUSSION

In an experiment with the cultured cells as heretofore described, it is desirable to infect all the cells at the time of inoculation and synchronize morphologic changes developed in them. In the present work, however, only a limited number of cells in the culture had distinctive nuclear change in an early stage of infection. Thereafter, the number of cells with cytopathic changes increased with the lapse of time. Consequently, a wide variation was observed in the morphologic alterations of cells at a given time. For this reason, the sequence of nuclear changes was determined by observing the time of their appearance in stained preparations. The nuclear changes thus observed appear to progress in nearly the same sequence as that described by Randall and Bracken,[14] using the hepatic cells of hamsters infected with EAV, and that by Goodpasture,[5] Scott *et al.*,[16] and Reissig and Melnick,[15] using other host cell systems infected with herpetic virus.

Coincidentally with the appearance of nuclear changes in the histologic preparations, nuclear alterations characterized by changes in the pattern of distribution of the chromatin became detectable under the electron microscope. These altered nuclei in an early stage of infection contained no recognizable viral particles. A few hours after this stage and shortly before the appearance of infective virus in the cellular phase of the cultures, viral particles made their appearance in the nuclei of the infected cells. Then these intranuclear particles, together with the intracytoplasmic and extracellular viral forms, appeared to increase in number parallel to a rise in infective titer in the culture.

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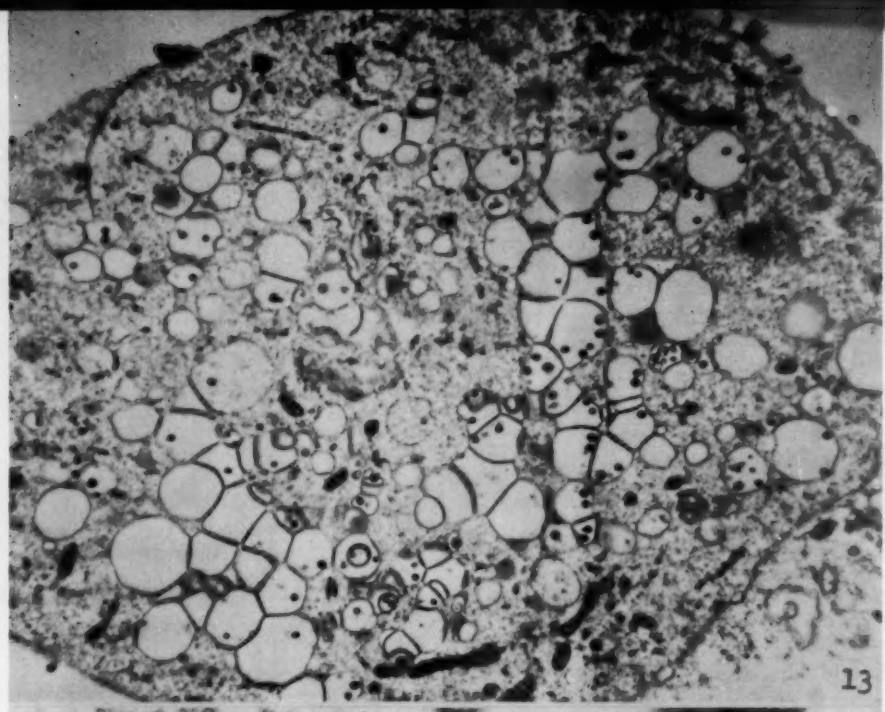
Fig. 11—Cells from a culture 14 hours after inoculation. Every nucleus contains a large central mass of dense osmiophilic material. This mass appears to correspond to the shrunken classical intranuclear inclusion in the stained thick section shown in the inset. Viral particles can be identified in no nuclei, whereas they are contained in the cytoplasmic vacuoles of 3 cells (arrows). Degeneration of the cytoplasm is prominent in all the cells. x 5,100.

Inset shows a thick section cut from the same block at a different level and stained with hematoxylin and eosin. The cells are in progressed stages of degeneration, exhibiting retraction of their cytoplasmic processes and marked increase in cytoplasmic basophilia. Most of their nuclei contain a classical type A inclusion with a clear halo. x 600.

Fig. 12—Part of a cell 36 hours after inoculation. Many clusters of viral particles are scattered throughout the nucleus. In these particles, hollow spheres that are apparently empty predominate, but a fairly large number of particles with a central dense dot are also present. The cytoplasm is filled with numerous vacuoles. Mitochondria are markedly swollen and their cristae are disintegrated. x 18,000.



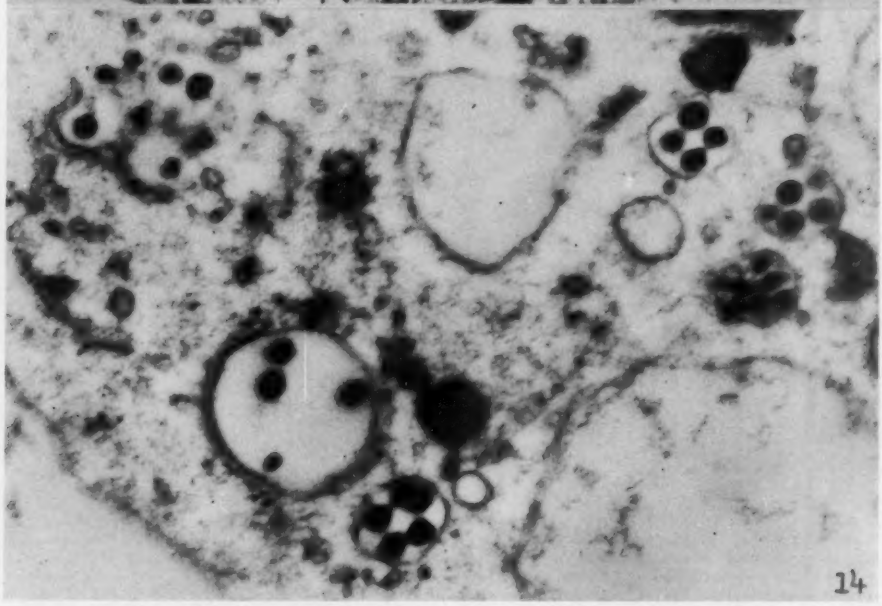
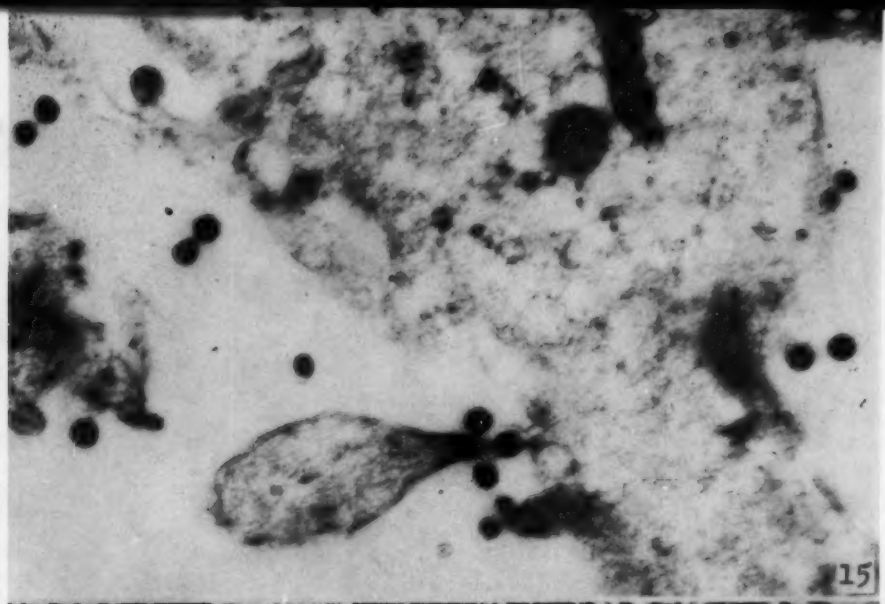


Fig. 13—Portion of the cytoplasm of a cell 24 hours after inoculation. Numerous cytoplasmic viral particles are present within the walled vacuoles. In the upper right corner of the picture, 3 dense extracellular particles are also seen on the external surface of the cell. x 9,600.

Fig. 14—Part of the cytoplasm of another cell 24 hours after inoculation. Cytoplasmic particles are shown at high magnification. Uniformly dense bodies without evidence of internal structure predominate. The wall of a particle-containing vacuole at the lower left is apparently double. x 39,600.



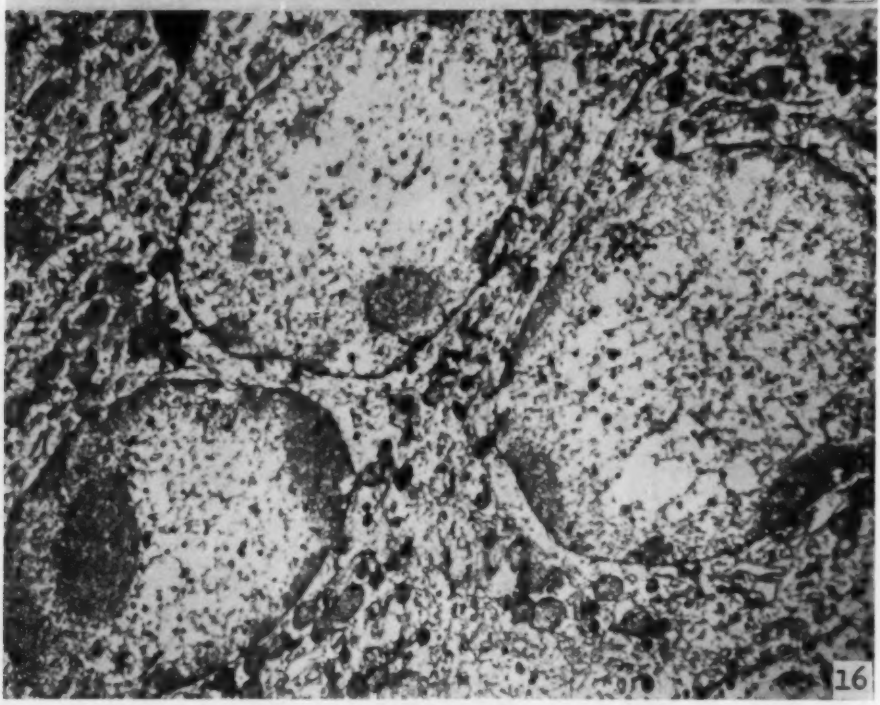
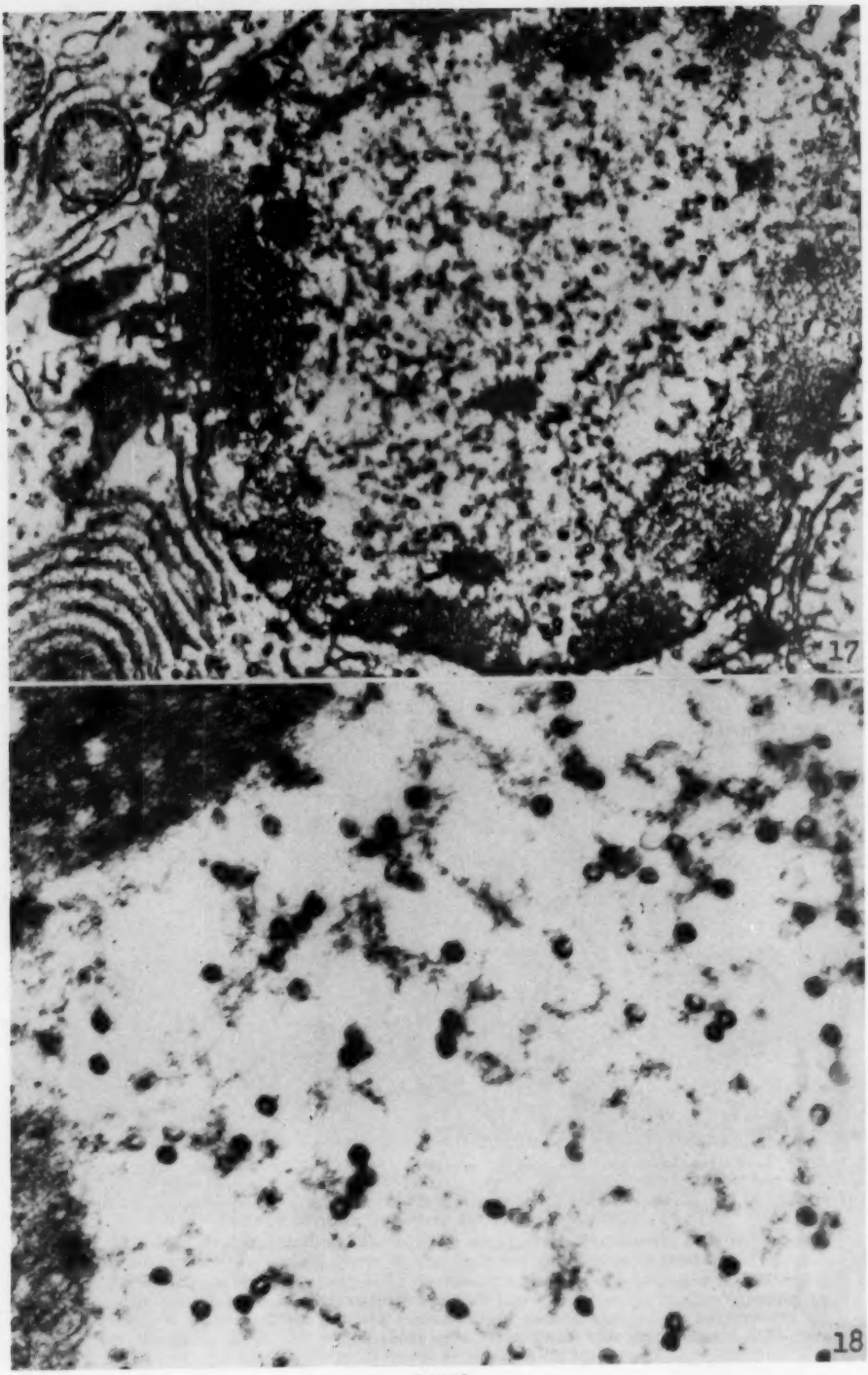


Fig. 15—Extracellular particles seen in a section from a culture 24 hours after inoculation. They are found outside the cell, lying free together with cell debris or attaching to the external surface of the cell, and do not differ in size and morphologic properties from the cytoplasmic particles shown in the preceding picture. x 36,000.

Figures 16 to 18 are electron micrographs taken of thin sections prepared from the livers of hamsters infected with the Kentucky D strain and killed in extremis.

Fig. 16—An area of a liver section including nuclei and portions of the cytoplasm of 3 parenchymal cells. In these nuclei, margination of chromatin and displacement of the nucleoli are evident, and a clear central zone in which viral particles are distributed at random can be seen. The clear central zone apparently corresponds to the site of an inclusion body in the histologic preparation as shown in figure 5. x 8,400.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The intranuclear viral particles identified in the present work agree roughly in size and appearance with those described by Bracken and Norris[1] in the hepatic cells of hamsters infected with the same virus. Their general configuration also resembles those described by previous authors[4,10,11,15,22] for some other animal viruses. They were usually randomly scattered within the nucleus and revealed no crystalline arrangement as simulated frequently by adenovirus,[7,9,12] herpes simplex virus,[13] papilloma virus,[19] and infectious canine hepatitis virus.[21] Cytoplasmic particles differed from intranuclear ones in that they were larger in size and generally had contents so electron-dense that their internal structure was hardly resolved. Although no exact relationship between the intranuclear and the cytoplasmic particles was established in the present investigation, it is conceivable, as has been postulated by Watrach *et al.*[22] for the virus of avian infectious laryngotracheitis, that the intranuclear particles represent an early stage of virus development. This interpretation is supported by the observations that a rise in virus titer in both the cellular and fluid phases of the culture appeared to be more closely related to the time of appearance of the cytoplasmic particles than to that of the intranuclear particles, and that the extracellular particles consisted exclusively of those particles which were morphologically identical with the cytoplasmic particles.

Cytoplasmic virus or virus-like particles surrounded by a membranous sac, similar to those observed in the present work, have been found in the electron microscopic studies of the virus associated with the Lucké tumor,[4] salivary gland virus,[10] herpes simplex virus,[13] and a cell-free induced leukemia of the mouse.[3] It has been suggested that these particles are in the process of egress from the nucleus into the extracellular space by way of the cytoplasmic canalicular system[10] or by the mechanism of reduplication of the nuclear membrane.[13] If this is the case for the virus studied in the present work, it would seem suitable to explain an early virus release which might occur before the beginning of disruption of infected cells. However, the state of viral particles in transit from the nucleus into walled vacuoles in the cytoplasm or to outside the cell has never been manifested.

In agreement with the observation of Bracken and Norris,[1] no structures definitely identifiable as intranuclear inclusion bodies were seen with the electron microscope, except for cultured cells in advanced stages of the degenerative process. Similar to findings by others[11,15] who worked with herpetic virus, the area occupied by an inclusion body was found to correspond to the clear central zone of the nucleus. The effect of fixatives (Zenker's acetic acid fluid, and buffered osmium tetroxide) on the appearance of inclusion bodies in the stained preparations, as pointed out by Reissig and Melnick,[15] was recognized, in some degree, by the present authors. However, it seems unreasonable to regard such effect of fixatives as the only factor responsible for the morphologic difference between the inclusions observed by light and electron microscopy. It should not be overlooked that sections are cut much thicker for light microscopy. Moreover, the authors are attracted to the possibility that, in addition to the osmiophilic amorphous material and viral particles, some other substance which is stainable

Legends for Illustrations on Opposite Page

Fig. 17—Part of another hepatic parenchymal cell showing typical nuclear changes. Nuclear chromatin is found in the form of large dense clumps adhering to the nuclear membrane. A clear central zone of the nucleus is occupied by numerous viral particles, among which hollow spheres predominate, and by a small amount of amorphous material. Rough-surfaced elements of the endoplasmic reticulum are prominent in the cytoplasm. x 16,200.

Fig. 18—Portion of the nucleus of another hepatic parenchymal cell. Various types of viral particles, which are similar to the nuclear forms observed in the cultured cells, can be distinguished. Large clumps of marginated chromatin are seen in the upper and lower left corners. x 30,000.

with eosin might play a role in the formation of inclusions.

Previous workers [2, 6, 16, 23] have indicated that the intranuclear herpetic inclusions in late stages contain no nucleoprotein and, presumably, no virus. In this connection, it is interesting that cultured cells in later stages of degeneration due to EAV apparently contained no viral particles in their nuclei, in spite of the presence of intranuclear structures reminiscent of shrunken type A inclusion bodies.

The results obtained from experiments with 2 strains of EAV, Kentucky D and the H 45, were similar with respect to their cytopathic effect as well as the morphology and distribution of viral particles.

SUMMARY

The morphologic changes occurring in horse kidney cells in monolayer cultures and in hepatic cells of hamsters following infection with equine abortion virus (EAV) were studied with the light and electron microscopes. In the cultured cells, a comparison was also made between the cellular alterations observed sequentially and the amount of infective virus detectable in the cultures at different hours after virus inoculation.

In infected cultured cells, nuclear changes characterized by disturbance of the chromatin pattern began to be observed under both the light and electron microscopes 3 hours after inoculation, a few hours before the appearance of viral particles within the nucleus or a rise in titer of cell-associated virus. Thereafter, the accumulation of inclusion material within the nucleus; the appearance of intranuclear, intracytoplasmic, and extracellular viral particles; and degenerative changes of the cytoplasmic organelles were observed. All these increased with the lapse of time, concomitant with the increase in amount of infective virus in both the cellular and fluid phases of the cultures. Electron microscopy failed, in most of the infected cells, to visualize any particular intranuclear structures suggestive of the inclusion bodies seen with the light microscope. The site of an inclusion body was found to correspond to the clear central zone of the nucleus in which viral particles were present. In the nuclei of cells in late stages of infection, a large, reticulated, central mass, reminiscent of a shrunken classical inclusion body, was often observed, but it usually contained no viral particles. Intranuclear viral particles were round to oval and approximately 95 mμ in diameter. Some of these particles had a dense inner body averaging 35 mμ in diameter. Intracytoplasmic and extracellular particles were similar in size and configuration, averaging about 115 mμ, and had contents of uniformly high electron-scattering power without evidence of internal structure. The majority of the cytoplasmic particles lay within walled vacuoles.

In hepatic parenchymal cells of infected hamsters, intranuclear, intracytoplasmic, and extracellular viral particles, similar in size and appearance to those observed in the cultured cells, were readily identified. Structures identifiable as inclusion bodies were not found.

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SUMMARIO IN INTERLINGUA

Microscopia Electronic de Virus de Aborto Equin

Le alterationes morphologic que occurre in cellulas de ren equin in cultural monostratal e in cellulas hepatic de hamsters post infection con virus de aborto equin (VAE) esseva studiate per medio de microscopia luminose e electronic. In le culturate cellulas, esseva etiam effectuate un comparation inter le alterationes cellular observate sequentialmente e le quantitate de infective virus detegibile in le culturas a varie horas post le inoculation del virus.

In inficite cellulas cultural, alterationes nuclear characterisate per perturbation del configuration chromatinic comenciava esser observabile tanto in microscopia luminose como etiam in microscopia electronic 3 horas post le inoculation, pauc horas ante le apparition de particulas de virus intra le nucleo o un augmento del titro de virus a association cellular. Postea, le accumulation de material de inclusion intra le nucleo; le apparition de particulas virusal intranuclear, intracytoplasmatic, e extracellular; e alterationes del organellos cytoplasmatic esseva observate. Omne iste constatationes se intensificava in le curso del tempore, in concomitantia con le augmento del quantitate de virus infective in le phases cellular e liquide del culturas. In le majoritate del inficite cellulas, le microscopio electronic non succedeva a render visibile ulle structuras intranuclear apte a suggerer le corpores de inclusion que esseva visibile con le microscopio a lumine. Esseva trovate que le sito de un corpore de inclusion correspondeva al clar zona central del nucleo in que particulas de virus esseva presente. In le nucleos de cellulas in tardive stadios de infection, un grande, reticulate massa central—simile in apparentia a un contractionate corpore de inclusion classic—esseva observate frequentemente, sed usualmente illo contineva nulle particulas de virus. Particulas de virus intranuclear esseva ronde o oval e approximativemente 95 mμ in diametro. In certe casos, iste particulas habeva un dense corpore interior con un diametro promedie de 35 mμ. Particulas intracytoplasmatic e extracellular esseva simile in dimension e configuration. Al promedia lor diametro esseva circa 115 mμ, e lor contento possedeva uniformemente un alte potentia de dispersion de electrones, sed nulle structura interne esseva evidente. Le majoritate del particulas cytoplasmatic se trovava al interior de parietes vacuolar.

In cellulas de parenchyma hepatic ab inficite hamsters, particulas virusal intranuclear, intracytoplasmatic, e extracellular—simile in largor e apparentia a illos observate in le culturate cellulas—esseva prestemente identificabile. Structuras a identificar como corpores de inclusion non esseva trovate.

Relationships Between Eastern and Western Equine Encephalomyelitis Viruses as Demonstrated by the Hemagglutination-Inhibition Antibody Response of Experimentally Infected Chickens

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SEROLOGIC and immunologic relationships between eastern and western equine encephalomyelitis (EEE and WEE) viruses have been previously investigated using a number of methods. Ten Broeck and Merrill [10] could not detect any cross reactions with the neutralization test in examining WEE- and EEE-immune horse serums. Quite recently, Parks and Price,[8] using day-old chicks as test animals for neutralization studies, substantiated these early observations of Ten Broeck and Merrill; *i.e.*, EEE and WEE were immunologically distinct.

Evidence suggesting immunologic relationships between EEE and WEE viruses was found with the complement-fixation test by Havens *et al.*[5] and Howitt.[7] Using the hemagglutination-inhibition (HI) test and employing hyperimmune mouse serum, Casals and Brown were able to demonstrate cross reactions between EEE and WEE viruses as well as other members of group A Arbor viruses.[2]

The work reported here was undertaken to investigate the serologic relationship between EEE and WEE viruses as demonstrated by the HI antibody response of experimentally infected chickens. The work entailed inoculation of WEE-immune chickens with EEE virus, and vice versa, and determination of their HI antibody response. In addition, the response of WEE- and EEE-immune birds to simultaneous infection with EEE and WEE viruses was studied.

MATERIALS AND METHODS

Viruses.—Two strains of EEE virus were used: the V-52 and Cambridge strains, both isolated from naturally infected horses in Maryland. The V-52 strain was isolated in 1956, and had undergone 4 mouse brain passages prior to inoculation into the experimental chicks. The Cambridge strain, isolated in 1958, had 1 chicken embryo passage, 2 passages in day-old chicks, and 2 mouse brain passages prior to inoculation. The WEE virus used was the $L_{2}34_{8}$ strain* and was subjected to 1 chicken embryo and 5 mouse brain passages prior to inoculation.

The mice employed in the original isolation and subsequent passages were 21-day-old Swiss Albinos and had been inoculated intracranially (i.c.). Chicken embryo passages were accomplished by inoculating 6- to 8-day-old fertile chicken eggs by the allantoic route and by harvesting whole embryos and fluids for subsequent transfers. Day-old chicks were inoculated subcutaneously and blood samples were obtained when chicks were moribund. Plasma from these dying birds served as passage material. Detailed information on the passage of WEE virus was not available.

Inoculation of Chickens.—Approximately equal numbers of Single Comb White Leghorn (SCWL) and New Hampshire (NH) chickens, totaling 35, were used in these experiments. These birds were purchased locally when 1 day old and housed in brooders until 3 weeks old, at which time they were inoculated. In all birds, the 0.1-ml. inoculum was administered subcutaneously. Both initial and re-exposure inoculums contained approximately 10^{4} mouse $l.d._{50}$ of the respective virus. The chickens were initially separated into 2 groups. Later, a third group was formed from EEE- and WEE-immune chickens selected from the first 2 groups. Each group was equally composed of the SCWL and NH breeds. Additional birds of the same age were used as controls for the various experiments.

Group 1.—The birds in group 1 were inoculated initially with WEE virus. Approximately 100 days later, 6 of the birds from this group were re-inoculated with WEE virus, while the others were inoculated with EEE virus.

Group 2.—Birds of the second group were initially inoculated with EEE virus; 4 birds were re-inoculated with EEE virus approximately 100 days later, while others were inoculated with WEE virus.

Group 3.—Birds from group 3 were inoculated simultaneously with EEE and WEE viruses approximately 100 days after initial inoculation with WEE or EEE virus.

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* Received from Dr. Robert Kissling, Communicable Disease Center, USPHS, Montgomery, Ala.

Blood samples were obtained from birds at various intervals following inoculation, and the serum was stored at −20 C. until the HI test could be performed.

Serologic Tests.—The method of hemagglutinin preparation was the same as that described by Chanock and Sabin for WEE.[3] In pilot hemagglutination (HA) and HI tests, goose erythrocytes were tested in parallel with erythrocytes from 1-day-old chicks. The sensitivity of goose erythrocytes to agglutination by Arbor viruses had previously been reported.[4,5] In these preliminary tests, serums were extracted with kaolin to remove nonspecific inhibitors.

The methods finally used for HA and HI tests were essentially those described by Clarke and Casals.[4] Modifications of these techniques have been previously described.[3,6] Serums were extracted with acetone, initially diluted 1:10, and titrated in fourfold steps through a final dilution of 1:10,240, except where otherwise indicated. The titer is reported as the reciprocal of the highest serum dilution giving complete inhibition of hemagglutination, using 8 units of antigen.

RESULTS

In initial attempts to utilize goose erythrocytes in HA and HI tests, the results were not entirely satisfactory. The HA titers of the same antigen aliquots were usually higher with goose erythrocytes than with those from day-old chicks. However, HA titrations with goose cells yielded end points which were subject to fluctuation and titer loss during incubation of the definitive HI test. Thus, an antigen diluted so as to contain 8 units (based on preliminary HA titration) would drop to 1 or 2 units in the HI tests of serum specimens. As a result, the HI test became overly sensitive, resulting in low-titer, false-positive reactions. Further studies indicated that these unsatisfactory results were compounded by using goose erythrocytes and kaolin-extracted serum in the HI tests. The most satisfactory results, therefore, were obtained with acetone-extracted serum and using day-old chick erythrocytes.

None of the preinoculation serums from any of the birds had detectable HI antibody for either EEE or WEE virus.

All chickens given initial doses of WEE virus had demonstrable HI titers for WEE

TABLE 1—Hemagglutination-Inhibition Antibody Response of Group I Chickens to Western Equine Encephalomyelitis (WEE) and Eastern Equine Encephalomyelitis (EEE) Viruses

Chicken No.	HI antigen used	Days following inoculation with WEE virus						Days following reinoculation *				
		0	7	14	21–22	49	102–110	0	5	16	30	42–44
EEE HETEROLOGOUS CHALLENGE, V-52 STRAIN												
	WEE	0**	160	640	2,560	2,560	2,560	2,560	2,560	.....	.....	2,560
670	EEE	0	0	0	0	0	0	0	160	.....	.....	640
	WEE	0	2,560	640	640	640	2,560	2,560	640	.....	.....	640
671	EEE	0	0	0	0	0	0	0	640	.....	.....	640
	WEE	0	40	160	160	40	160	160	160	.....	.....	640
674	EEE	0	0	0	0	0	0	0	640	.....	.....	640
	WEE	0	160	640	640	640	640	640	640	.....	.....	640
675	EEE	0	0	0	0	0	0	0	640	.....	.....	640
EEE HETEROLOGOUS CHALLENGE, CAMBRIDGE STRAIN												
	WEE	0	40	160	640	.....	160	160	.....	2,560	640	640
821	EEE	0	0	0	0	.....	0	0	.....	2,560	2,560	2,560
	WEE	0	160	640	2,560	.....	640	640	.....	640	2,560	2,560
823	EEE	0	0	0	0	.....	0	0	.....	640	640	640
WEE REINOCULATION												
	WEE	0	160	640	640	640	640	640	640	.....	.....	640
622	EEE	0	0	0	0	0	0	0	0	.....	.....	0
	WEE	0	40	160	40	10	40	40	40	.....	.....	10
637	EEE	0	0	0	0	0	0	0	0	.....	.....	0
	WEE	0	40	160	40	10	40	40	40	.....	.....	10
672	EEE	0	0	0	0	0	0	0	0	.....	.....	0
	WEE	0	160	640	640	640	640	640	640	.....	.....	160
673	EEE	0	0	0	0	0	0	0	0	.....	.....	0
	WEE	0	2,560	2,560	2,560	2,560	640	640	.....	2,560	.....	.....
824	EEE	0	0	0	0	0	0	0	.....	0	.....	.....
	WEE	0	160	640	640	640	640	640	.....	640	640	640
818	EEE	0	0	0	0	0	0	0	.....	0	0	0

..... = not done.

* 103 to 111 days following initial exposure.

** Reciprocal of serum dilution giving complete inhibition of hemagglutination.
0 = negative response at the 1:10 dilution of serum.

TABLE 2—Hemagglutination-Inhibition Antibody Response of Group 2 Chickens to Western Equine Encephalomyelitis (WEE) and Eastern Equine Encephalomyelitis (EEE) Viruses

Chicken No.	HI antigen used	Days following inoculation with WEE virus						Days following reinoculation*				
		0	7	14	21–22	49	102–110	0	5	16	30	42–44
WEE HETEROLOGOUS CHALLENGE												
V-611**	EEE	0	160	640	640	640	640	640	640	.....	.....	640
	WEE	0	0	0	0	0	0	0	160	.....	.....	2,560
V-648	EEE	0	160	160	160	40	160	160	160	.....	.....	160
	WEE	0	0	0	0	0	0	0	160	.....	.....	640
C-804	EEE	0	160	160	640	.....	640	640	.....	640	160	.....
	WEE	0	10	10	10	.....	10	10	.....	640	640	.....
C-806	EEE	0	640	640	640	.....	160	160	.....	.....	640	640
	WEE	0	0	0	0	.....	0	0	.....	.....	640	640
EEE REINOCULATION, V-52 STRAIN												
V-610	EEE	0	160	160	160	10	10	10	40	.....	.....	160
	WEE	0	0	0	0	0	0	0	0	.....	.....	0
V-845	EEE	0	40	160	160	40	40	40	40	.....	.....	40
	WEE	0	0	0	0	0	0	0	0	.....	.....	0
C-802	EEE	0	640	640	2,560	.....	160	160	.....	160	.....	.....
	WEE	0	0	0	10	.....	20	20	.....	160	.....	.....
C-808	EEE	0	2,560	640	2,560	.....	640	640	.....	.....	.....	.....
	WEE	0	10	10	0	.....	40	40	.....	.....	.....	.....

..... = not done. Serum from bird C-802 was diluted in twofold steps.
* = challenged 103–111 days following initial exposure.
**V = indicates the chicken was inoculated with EEE virus strain V-52; C = indicates the chicken was inoculated with EEE virus strain Cambridge.

by the seventh day following inoculation (table 1). These titers were from 1:160 to 1:2,560, in most cases, for at least 100 days. However, HI antibody for EEE virus was not detected in the serums of any of these birds during this period. Birds from this group, subsequently inoculated with EEE virus, responded by producing HI antibody for EEE. In 1 of these 6 birds (821), there was a restimulation of WEE antibody as indicated by a 2-tube increase in HI titer. The birds in this group which were reinoculated with WEE virus did not respond markedly; *i.e.*, there was neither significant increase of WEE antibody or development of antibody to EEE.

The results from birds in group 2 are listed (table 2). The HI antibody for EEE virus appeared by the seventh day and was detectable in all birds for at least 100 days, which closely parallels the pattern of group 1 birds for WEE virus. However, in 3 of the 8 birds tested (802, 804, 808), WEE virus HI titers appeared, though at a low order, by the seventh day in 2 of the birds and by the 21st day in the other bird. These low titers persisted for at least 100 days. Interestingly, the 3 birds whose serums cross-reacted with WEE virus had all been given the Cambridge (Md.) strain of EEE virus. Birds in this group that were inoculated with WEE virus after the initial EEE virus inoculation developed HI antibody to WEE without significantly restimulating EEE antibody.

Of the 4 birds initially inoculated with EEE virus and then reinoculated with EEE virus, only 1 (610) developed an anamnestic response. Bird 802 had no change in HI titer for EEE, but the heterologous WEE titer was found to have risen eightfold.

At the time that the group 1 and 2 birds were reinoculated or their immunity was challenged, equal numbers of normal control birds of the same age were inoculated with either EEE or WEE virus. The HI antibody titers were detected only for the homologous antigen, and no cross reactions were found.

As previously stated, group 3 birds, consisting of those selected from groups 1 and 2, were immune to either EEE or WEE virus. The results following simultaneous inoculation of these birds and control birds with EEE and WEE viruses are shown (table 3). Birds immunized against 1 virus and subsequently inoculated with both responded serologically only to the virus to which they had not previously been immunized. In control birds inoculated with both viruses, independent HI antibody development to EEE and WEE virus was manifested. In 2 birds (837, 843), HI antibody response to the 2 viruses was unequal. The significance of this effect is difficult to evaluate, since the birds were not subjected to blood

sampling after 15 days, thus precluding detection of higher titers.

DISCUSSION

It is evident from the results that chickens inoculated initially with WEE virus responded serologically in a manner similar to those inoculated with EEE; *i.e.*, there was a rapid production of specific HI antibodies which persisted at a high level for at least 100 days. The only exception noticed was in the case of 3 nonimmune birds inoculated with EEE virus which produced a HI titer of a low order for WEE, in addition to the homologous antibodies.

TABLE 3—Hemagglutination-Inhibition Antibody Response of Normal and Immune Group 3 Chickens Inoculated Simultaneously with Eastern Equine Encephalomyelitis (EEE) and Western Equine Encephalomyelitis (WEE) Viruses

Chicken No.	HI antigen used	Days following inoculation 0	5	7	15
INOCULATED WITH WEE 111 DAYS PREVIOUSLY					
	WEE	640	640	2,560	.....
638	EEE	0	640	640	.....
	WEE	160	640	640	.....
639	EEE	0	0	640	.....
	WEE	160	160	640	640
816	EEE	0	640	2,560	2,560
INOCULATED WITH EEE (V-52) 111 DAYS PREVIOUSLY					
	EEE	40	40	40	.....
608	WEE	0	160	640	.....
	EEE	40	160	160	.....
609	WEE	0	40	640	.....
CONTROLS					
	EEE	0	0	640	160
852	WEE	0	0	160	40
	EEE	0	640	640	160
837	WEE	0	0	10	10
	EEE	0	2,560	2,560	160
838	WEE	0	160	160	40
	EEE	0	0	10	10
843	WEE	0	40	160	160
	EEE	0	160	160	.....
656	WEE	0	0	40	.....

..... = not done.

Immune birds that were reinoculated either with EEE or with WEE virus responded serologically to the virus to which they were susceptible. In general, there were few anamnestic reactions attributable to a heterologous or homologous stimulus in previously immunized birds.

It would appear from the limited number of birds given both viruses that antibody development for WEE and EEE occurred independently and that there was no obvious evidence of virus interference. The results of these experiments, except for a few cases, suggest that EEE and WEE viruses are not closely related. These results are in agreement with those of early workers who used cross-protection tests to differentiate EEE and WEE viruses and the more recent evidence of the independence of EEE from WEE obtained by Parks and Price.[8] Differences between results cited and those of Casals and Brown,[2] who reported cross-reactions between EEE and WEE virus, may be attributed to the species of animals used to produce immune serums, and to the method of immunization.

SUMMARY

Three-week-old Single Comb White Leghorn and New Hampshire chickens developed relatively high HI antibody titers by the seventh day following inoculation with approximately 10^4 mouse $l.d._{50}$ of western or eastern equine encephalomyelitis (WEE and EEE) viruses. These titers persisted for at least 100 days and, during that time, a low level of cross reaction was observed only in the case of birds inoculated with EEE (Cambridge strain) virus.

Challenge doses of EEE or WEE viruses, given to homologous- or heterologous-immune birds, produced only a few instances of heterologous antibody stimulation.

Simultaneous inoculations made with WEE and EEE viruses in normal and immunized birds generally stimulated the production of HI antibodies for each virus.

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SUMMARIO IN INTERLINGUA

Le Relationes Inter Virus de Encephalomyelitis Equin del Est e Virus de Encephalomyelitis Equin del West, Demonstrate per le Responsa de Anticorpore a Inhibition de Hemagglutination in Experimentalmente Inficite Gallinas

Gallinas de tres septimanas de etate del racias Leghorn Blanc a Cresta Solitari e New Hampshire disveloppava relativemente alte titros de anticorpore a inhibition de hemagglutination al fin de septe dies post le inoculation de 10^4 DL_{50} mus de virus de encephalomyelitis equin del west e de virus de encephalomyelitis equin del est (EEW e EEE). Iste titros persisteva durante al minus 100 dies. In le curso de iste periodo, basse nivellos de reaction cruciate esseva notate solmente in le caso de aves inoculate con virus de EEE (racia Cambridge).

Doses de provocation de virus de EEE o EEW, administrate a aves con immunitate homologe o heterologe, produceva solmente un basse numero de casos de stimulation de anticorpore heterologe.

Inoculationes simultanee de virus de EEW e de EEE in aves normal e in aves immunisate stimulava in general le production de anticorpore a inhibition de hemagglutination pro cata un del duo typos de virus.

The Isolation and Propagation of a Naturally Occurring Turkey Lymphoid Tumor by Cellular Transplants

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IT HAS BEEN KNOWN for some time that turkeys are susceptible to lymphoid tumors.[2-5] The striking similarity of these tumors to those found in chickens with visceral lymphomatosis (VL) has resulted in the application of the same term to both conditions. It appears logical at this time to assume that in the turkey, as in the chicken, the neoplasm is induced by a virus,[1] although circumstantial evidence is yet to be confirmed. To our knowledge, no reference to the isolation and propagation of a naturally occurring lymphoid tumor of the turkey has appeared in the literature.

MATERIALS AND METHODS

Four lymphoid tumors obtained from turkeys of 4 different flocks were selected for study. The primary tumor used in this investigation was collected from a 12-week-old female turkey examined at the Ohio Agricultural Experiment Station. At the time the turkey was examined, the liver was greatly enlarged. Numerous gray-white tumors were present on the surface and throughout the organ. Gross examination of other viscera, including the spleen, was negative for these lesions.

The turkeys used in these experiments were from 2 sources. Half were purchased as day-old unsexed birds from a commercial Ohio hatchery; the remainder were obtained from the experiment station flock. Chickens were purchased as day-old cockerels from a local pullorum-free hatchery.

Suspensions of tumor cells for serial transfer were prepared by trypsinization. The tumors were harvested, minced in a sterile beaker using scissors, and placed in a 250-ml. Erlenmeyer flask containing a plastic-coated bar magnet. A 0.5 per cent solution of trypsin 1:250 (Difco) was prepared, using Hanks' balanced salt solution. All solutions contained penicillin at levels from 100 to 2,500 units per milliliter, and dihydrostreptomycin at the rate of 10 milligrams per milliliter. These solutions were adjusted to a pH of 7.4, using a sterile 4.0 per cent solution of sodium bicarbonate.

The trypsin solution was added to the minced tissue in the proportion of 4:1 and processed on a magnetic stirring device* until digestion was accomplished, which usually required about 40 minutes at room temperature. The resulting suspension of cells was strained through fine cheesecloth (gauze) into 50-ml. centrifuge tubes and spun for 5 minutes at 750 r.p.m. in a refrigerated centrifuge at 5 C. The supernatant fluid was discarded and the cellular sediment resuspended in Hanks' solution without trypsin. After repeating the centrifugation, the sediment was resuspended in Hanks' solution at a rate of 1:10 for inoculation into the deep pectoral muscles of the experimental birds.

Inoculations of tumor cells into young mice, rabbits, and guinea pigs were made intramuscularly in the scapular region. Intrauterine inoculation of tumor cells into pregnant guinea pigs was accomplished by performing a laparotomy and injecting suspensions of cells into the fetuses in the uterine horns.

The preparation of tissues for histologic examination included use of 10 per cent formalin as a fixative and hematoxylin and eosin for staining.

RESULTS

The liver of the original donor turkey was reduced in a TenBroeck tissue grinder, following the method outlined by Burmester[1] but using Hanks' solution as a vehicle. The resulting cellular suspension was inoculated intramuscularly in 0.5-ml. amounts into 104 turkeys (2 weeks old) and 25 chickens (7 days old). Although none of the chickens developed tumors, 8 of 100 surviving turkeys developed neoplasms at the site of inoculation within 21 days after injection. The tumor-like lesions in these birds were very firm and, unlike typical lymphoid tumors, developed areas of central necrosis. Cell-free extracts of these tumors, inoculated into the breast muscles of 20 turkeys, did not reproduce similar lesions. After removing the necrotic tissue, the tumor was reduced by trypsinization and reinoculated intramuscularly into 19 turkeys, 1 week old. This produced lymphoid tumors in all turkeys by the seventh postinoculation day.

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* Magnestir, manufactured by Chicago Apparatus Co., Chicago, Ill.

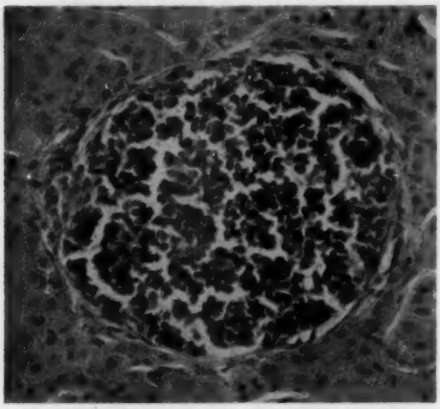

Fig. 1—Metastasis in liver of turkey inoculated intramuscularly with cells of the primary turkey tumor. H & E stain; x 290.

Surviving birds used for the third passage were killed on the eleventh day. Cell suspensions of tumors harvested from them were inoculated into both turkeys and chickens. Although all 18 of the inoculated turkeys developed tumors at the site of inoculation in 11 days, only 1 of 12 chickens developed a neoplasm. The tumors in the turkeys were necrotic, but that in the chickens was not. When the chicken tumor material was reinoculated into 25 turkeys and 25 chickens, the resulting neoplasms in 20 chickens were again free of necrosis, while those in each of the 25 turkeys had extensive central necrosis.

After the fourth serial passage in turkeys, the transplants produced death in some birds as early as the sixth postinoculation day. Metastases were found in the livers of those which survived until the eighth day (fig. 1). The histologic pattern of the metastases was similar to that in naturally occuring lymphomatosis in turkeys described by Simpson *et al.*[5]

After the initial isolation was accomplished, this neoplasm was carried through serial passage in turkeys without difficulty, using intramuscular transplants. Cells harvested from the third, fifth, and seventh passages were successfully transplanted into the breast muscles of chickens. After the seventh serial passage in turkeys, all attempts to grow the neoplasms in chickens were unsuccessful.

Repeated attempts were made to transmit the tumor to turkeys, using cell-free inoculums. Tumor extracts were prepared and administered following the procedure successfully used by Burmester.[1] Although more than 300 turkeys were used, no neoplasms developed in birds held for as long as 270 days.

Although the heterotransplantation of this neoplasm into chickens was accomplished, attempts to grow the cells in 12 young white mice, 8 rabbits (20 hours old), and 7 guinea pigs (24 hours old) were unsuccessful. Intrauterine inoculation of viable cells into 4 pregnant guinea pigs was also attempted without success.

Similar techniques were used in attempts to transplant material from the other 3 turkey tumors, obtained from birds with field cases, in young turkeys in the laboratory. In no instance was transplantation accomplished with these 3 tumors, even though 75 to 104 turkeys were used for each.

DISCUSSION

The primary neoplasm used in this investigation was adapted for cultivation in young turkeys by inoculating cells of the original tumor into a considerable number of birds. The same technique was not successful with 3 similar tumors. It is a well-known but sometimes overlooked fact that strains of birds vary in susceptibility to established laboratory strains of VL agents. This may be a partial explanation for failure to transmit the other 3 tumors.

According to Greene,[3] all 4 attempts should have succeeded, since the tumors of VL are very prone to metastasis. According to Snell,[7] all 4 should have failed since the donor and recipient of the transplants were not from a highly inbred line of turkeys.

In the tumor which was successfully adapted to laboratory passage in turkeys, only material of the early passages could be transplanted into chickens. The virulence for turkeys appeared to increase, while that for the chicken decreased to the point where heterotransplantation from turkeys to chickens was no longer accomplished.

It is apparent that the initial isolation of cells poses the greatest problem in establishing new strains of tumor cells for laboratory transmission.

SUMMARY

Cells of a lymphoid tumor from a turkey with a field case of lymphomatosis were

successfully transplanted into 8 of 100 turkeys within 21 days but not into any of 25 chickens. Numerous serial passages of trypsinized cellular material were accomplished with increasing mortality in turkeys. Cells of the third, fifth, and seventh serial passages in turkeys were successfully transplanted into chickens, but cells of subsequent passages did not grow.

Repeated attempts to transmit the tumor to turkeys, using cell-free inoculums in 300 birds, were unsuccessful during the 270-day observation period.

Unsuccessful attempts were made to grow the cells in young mice, rabbits, and guinea pigs as well as in guinea pig fetuses in the uterus.

Attempts to adapt 3 other similar tumors to passage in laboratory turkeys were unsuccessful, using 75 to 104 turkeys per tumor.

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SUMMARIO IN INTERLINGUA

Le Isolation de Tumor Lymphoidic de Gallos de India de Occurrentia Natural e Su Propagation per Transplantas Cellular

Cellulas de un tumor lymphoidic occurrente naturalmente in un gallo de India esseva transplantate a bon successo in 8 ex 100 gallos de India intra 21 dies sed in nulle de 25 gallinas. Numerose passages in serie de trypsinisate material cellular esseva effectuate in gallos de India con crescente cifras de mortalitate. Cellulas del tertie, del quinte, e del septime passage serial in gallos de India esseva transplantate a bon successo in gallinas, sed cellulas ab subsequente passages non cresceva.

Repetite essayos de transmitter le tumor a gallos de India con le uso de inoculos acellular in 309 aves non succedeva in le curso del periodo de observation de 270 dies.

Remaneva sin successo le essayos de effectuar un crescentia del cellulas in juvene muses, conilios, e porcos de India o etiam in fetos de porco de India in utero.

Effortios de adaptar 3 altere simile tumores al passage in gallos de India laboratorial remaneva sin successo, con le uso de inter 75 e 104 gallos de India per tumor.

A Modified Microgel Diffusion Method and Its Application in the Study of the Virus of Duck Hepatitis

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In a preliminary study to develop some serologic methods for diagnosis of duck hepatitis virus (DHV), a simple, modified, microgel diffusion method was evolved. The procedure was found to be useful in the study of this virus as well as other viral and bacterial antigens.

Oudin [9] was the first to carry out a systematic investigation of the gel diffusion technique for the study of specific antigen-antibody reactions. He used a simple vertical diffusion method conducted in tubes; later, Oakley and Fulthrope [7] adopted the same method for double-diffusion work. Ouchterlony [8] and Elek [2] independently described a double-diffusion technique conducted in Petri dishes containing agar gel medium. Grasset *et al.*[3,4] developed a method which they called a rapid slide precipitin micro reaction, in which microscope slides were coated with a thin layer of agar and in which holes, 2 mm. in diameter and 4 mm. apart, were punched with a special cutting appliance. These holes were filled with approximately 4 ml. of specific reactants. The slides were then incubated in a sealed chamber with a water-saturated atmosphere. The precipitation lines appeared in 24 hours or less but could be observed only after an elaborate process of staining or by dark-ground illumination. They also reported that the lines of precipitation regressed after further incubation.

Mansi [6] described a rapid gel diffusion method in which he also used microscope slides coated with a thin layer of gelled agar medium containing methyl orange. A hexagonal pattern of cups, 2 mm. in diameter and 1.5 mm. apart, were cut in the medium. The slides were then incubated at 37 C., and the optimal time for observation of the precipitation lines for various antigen-antibody systems was recorded. He also observed that the flocculation lines regressed after further incubation. Dumbell and Nizamuddin [1] followed essentially the technique of Mansi [6] in the study of smallpox virus, except that they used slightly larger cups.

In the technique adopted in this study, attempts have been made to further simplify the procedure by dispensing with the use of any staining methods and by carrying out the test at room temperature and in normal atmosphere. No special cutting appliance was used for preparing the cups, and the composition of the gel medium was altered to make it more sensitive and useful for chicken and bovine serums. In the experimental work conducted so far, it has been observed that the flocculation lines did not regress after further incubation.

Methods and Materials

The agar medium was prepared by dissolving 1.0 Gm. of agar powder (Difco), 8.0 Gm. of sodium chloride, and 0.001 Gm. of thimerosal* in 100 ml. of 0.15 M phosphate-buffered saline solution (pH 7.2) and sterilized by autoclaving at 120 C. for 15 minutes. The buffered saline solution was prepared by mixing 12 ml. of 0.15 M solution of KH_2PO_4 and 28 ml. of 0.15 M solution of Na_2HPO_4 in 40 ml. of physiologic saline solution.

Several variations in the composition of the medium were tested, and the values listed were found to be most suitable for general use. The amount of agar in the medium can be reduced to 0.75 per cent, although the medium becomes softer, making it difficult to cut the cups in the medium. The pH can be altered from 6.8 to 7.2 without

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Taken in part from a thesis submitted by the senior author in partial fulfillment of the requirements for the M.S. degree.

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* Merthiolate, produced by Eli Lilly & Co., Indianapolis, Ind.

materially affecting the reaction. However, when chicken and bovine serums are used, a higher concentration of sodium chloride (8.0%) is particularly useful. Rabbit serums reacted at any salt concentration ranging from 0.85 to 12.0 per cent, although the flocculation lines appeared to be more distinct at 8.0 per cent than at other levels. Addition of indicator dyes such as methyl orange, Congo red, or methylene blue did not materially enhance the appearance of the precipitation lines and were not incorporated in the medium. The medium can be prepared without autoclaving, after boiling it in a water bath for 15 to 20 minutes. Freshly prepared liquid medium should be used directly for preparing the plates, since remelted agar gel is not as satisfactory as new mediums for the test.

Preparation of Slides.—Microscope slides without any scratches or fogging were cleaned by soaking overnight in 90 per cent alcohol and dried just before use. The pattern of cups to be prepared is first drawn on paper. A rectangular area, 3.0 by 2.5 cm., is marked on the paper, in the center of which the pattern to be cut is drawn. A square pattern of 1 central and 4 peripheral cups (fig. 1) is most suitable for general use, as it is convenient for labeling the cups as well as for observing the lines of precipitation. Various other patterns, such as triangular, quadrangular (fig. 2), hexagonal, or octagonal can be used, depending on the nature of the reactants and the purposes of study. A cup diameter of 4 mm. and a distance of 2 to 3 mm. between the cups proved to be most suitable in this study. A cup size of 5 mm. can also be used.

The microscope slide is placed over the paper and the 2 lines, 3 cm. apart, are drawn on the slide with a wax pencil. Narrow cellophane tape can also be used instead of wax lines. This will leave sufficient space (2.0 cm.) on either end of the slide for labeling the cups. One and one half milliliters of liquid medium (60–70 C.) is slowly layered onto the center area of a slide with a 5-ml. graduated pipette and allowed to solidify. The slide is then transferred to a Petri dish and stored in a refrigerator for about 2 hours to allow the agar to become firm before the pattern is cut.

Fig. 1—Microgel diffusion test (square pattern). The top and right side cups (A and B) were filled with unabsorbed hyperimmune rabbit serum; the central cup was filled with embryonal liver infected with duck hepatitis virus; and the bottom and left cups (C and D) were filled with normal rabbit serum. Notice the 2 lines of specific precipitation (1 and 2).

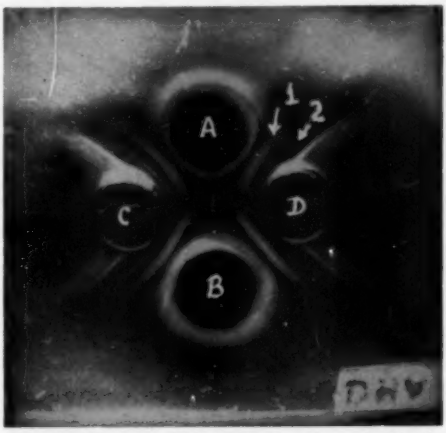

Fig. 2—Microgel diffusion test (4-cup pattern). The top and bottom cups (A and B) were filled with embryonal liver infected with duck hepatitis virus; the cups on either side (C and D) were filled with unabsorbed hyperimmune rabbit serum. Notice the different lines of precipitation. (Lines 1 and 2 are specific).

When the slide is taken from the refrigerator, the agar-coated area is placed over a paper containing the chosen cup pattern. Care should be taken that the cups are not cut too close to the edge of the block, as this will cause distortion as the moisture evaporates from the medium. With the help of a metal cork-borer of appropriate size, the patterns of cups are cut and the medium is removed from the cups with a Pasteur pipette by suction. These slides, if stored in a refrigerator (4 C.), can be used as long as 2 or 3 days after preparation.

Antigens.—The DHV used was strain R 85952, isolated in June, 1955, during an epizootic of this disease at a central Illinois farm.[5] The eighteenth chicken embryo passage of this strain was used to inoculate 8 day-old chicken embryos. After the death of the 8 embryos, the amnioallantoic fluid (a.a. fluid) and the livers were collected separately. These materials were used as antigens without pretreatment. Lungs and spleens of some of the infected chicken embryos were also used in a few trials. The a.a. fluids of uninfected chicken embryos of the same age were used as controls in the study.

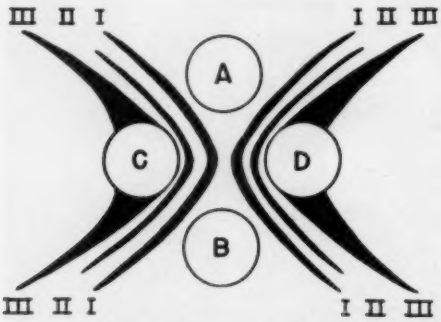

Fig. 3—Schematic diagram of microgel diffusion test shown in figure 2. Cups A and B contain antigen (infected chicken embryo liver tissue). Cups C and D contain untreated hyperimmune rabbit serum. Notice the 3 lines of precipitation (I, II, III). The third line was not evident with duck serum.

Serum.—Serum samples, obtained from Mallard ducks hatched at the Illinois Conservation Department and exposed to the same strain of DHV, were used during this investigation. Hyperimmune serum was prepared in a rabbit by 5 intravenous injections of infected a.a. fluid (3 1-ml. and 2 2-ml. doses) administered at intervals of 4 days. Serum samples from uninfected ducks and rabbits were used as controls.

Homotypic serum was prepared by treating the hyperimmune rabbit serum with the normal a.a. fluid or with a concentrated suspension of normal embryonal liver. By this treatment, the nonspecific flocculation reaction was eliminated.

Test Procedure.—The cups were filled with antigens or serum samples by means of finely drawn Pasteur pipettes. In the case of tissues, a small piece of the material was placed directly in the cup by means of a platinum loop, and the tissue piece was covered with a drop of sterile physiologic saline solution. The slides were incubated at room temperature in a normal atmosphere. Usually, when comparing several serum samples with a known antigen, the central cup in the square pattern was filled with the antigen, while the peripheral cups were filled with antiserum. The test was also carried out in the reverse way, with known antiserum and unknown antigens. A triangular or 4-cup pattern was used in comparing 2 unknown antigens with a known positive serum or for the study of 2 unknown serums with a known antigen. Although the lines of precipitation were observed even after a single filling of the cups, refilling the cups 3 or 4 times at intervals of about an hour enhanced the intensity of the flocculation lines. No reaction occurred between negative serum samples and the antigen, even when the cups were filled 16 times. No change in the pattern of the flocculation lines resulted from refilling except in increasing their intensity. Usually, 3 or 4 fillings were sufficient.

OBSERVATIONS

Lines of precipitation appeared within 18 to 24 hours and could be observed by indirect light. In some antigen-antibody systems, the reaction appeared within 4 hours, while in others it appeared only after 24 hours, depending on the rate of diffusion of the antigen-antibody system and the concentration of the reactants in the fluid. Generally, the slide was incubated for 48 hours at room temperature, after which it was transferred to a refrigerator for 3 or 4 days. When untreated hyperimmune rabbit serum and infected embryonal liver were used in the square pattern, 3 lines of precipitation were observed (fig. 1). The first line appeared after 4 hours and the second and third lines after 12 hours. The first and second lines were slightly concave, thicker in the middle, and tapering toward the periphery. The third line was close to the antibody cup. Sometimes, a faint fourth line appeared near the antigen cup after 18 hours. In the 4-cup pattern, the top and bottom cups (A and B) were filled with DHV-in-

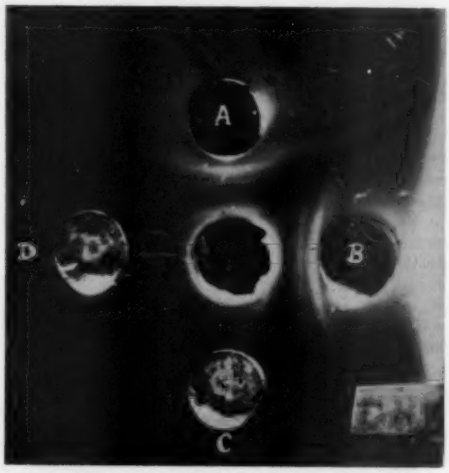

Fig. 4—Microgel diffusion test (square pattern) with absorbed hyperimmune serum. The cups at the top and right side (A and B) were filled with absorbed serum; the central cup was filled with embryonal liver tissue infected with duck hepatitis virus; and the bottom and left cups (C and D) were filled with normal rabbit serum. Only 2 specific lines of precipitation were present 12 hours after setting up the test. Notice their curvature and appearance.

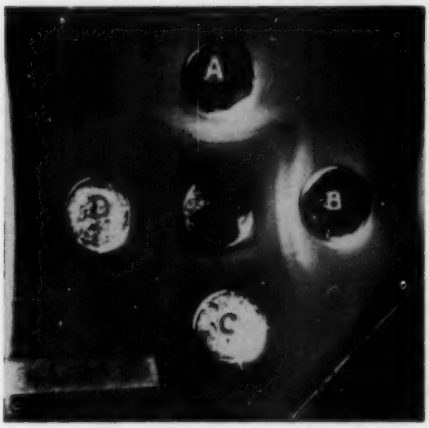

Fig. 5—The same test as in figure 4, after 24 hours. Notice the confluence of the 2 lines of precipitation from the adjoining cups (A and B). Cups A and B were filled with absorbed hyperimmune serum; the central cup was filled with embryonal liver tissue infected with duck hepatitis virus and cups C and D with normal rabbit serum.

fected liver; the cups on either side (C and D) were filled with hyperimmune rabbit serum (fig. 2, 3). The lines of precipitation were quite prominent, with those on one side joining the respective lines of the opposite side in the middle. With duck immune serum samples in the bottom cups (B and C) in a triangular pattern, and the infected liver in the top cup (A), only a single line of precipitation appeared on either side of the antigen cup, joining the opposite line in an obtuse angle. These lines, however, appeared after 24 hours and were not consistent.

When the test was conducted with absorbed hyperimmune rabbit serum, utilizing the square pattern and infected liver as the antigen, all other lines were eliminated except for the 2 that appeared after 4 hours and 12 hours, respectively (fig. 4). The lines from the adjoining cups became confluent in about 18 to 24 hours, forming a V-pattern (fig. 5). With infected a.a. fluids, an almost identical pattern of precipitation was obtained. Uninfected embryonal liver and a.a. fluid failed to develop any lines or precipitation with homotypic serum. None of the negative serum samples from rabbits or ducks was reactive.

When the central cup in a square pattern was filled with hyperimmune rabbit serum and the peripheral cups with infected chicken embryonal liver, only 1 line of precipitation appeared and all the 4 lines joined together to form a square pattern around the central cup (fig. 6). With the tissue culture antigen, the first line of precipitation appeared after 18 hours and became more intense after 24 hours. The second line, less defined, frequently appeared after 48 hours.

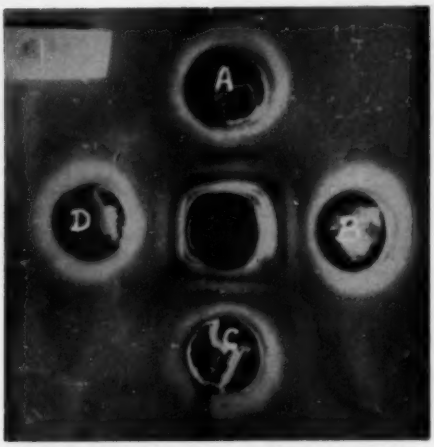

Fig. 6—Microgel diffusion test (square pattern). The central cup was filled with hyperimmune rabbit serum; the 4 peripheral cups (A, B, C, and D), with the same aliquots of embryonal liver tissue infected with duck hepatitis virus. Notice a single line of precipitation between the central and peripheral cups.

SUMMARY

The precipitation pattern obtained with a simplified method for carrying out microgel diffusion was studied with respect to the virus of duck hepatitis and its hyperimmune serum. This technique appeared to be useful in the study of chicken and bovine antiserums.

The test utilized agar placed on microscope slides. Wells were cut 4 mm. apart in the agar with a cork borer.

Visible reactions occurred in 4 to 24 hours at room temperature and could be readily observed in indirect light.

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SUMMARIO IN INTERLINGUA

Un Modificate Micromethodo a Diffusion in Gel, e Su Application al Studio del Virus de Hepatitis de Anate

Le configuration precipitational obtenite per medio de un simplificate micromethodo de diffusion in gel esseva studiate con respecto al virus de hepatitis de anate e su sero hyperimmun. Iste technica pareva utile in le studio de antiseros de gallina e bovin.

Le test utilisava agar placiate super porta-objectos microscopic. Puteos esseva secate a intervallos de 4 mm in le agar per medio de un forator de corco.

Reactiones visibile occurreva intra 4 a 24 horas al temperatura normal de interior e poteva esser observate facilemente in lumine indirecte.

Observations on the Disturbance of Respiration of Cattle, Horses, Sheep, and Dogs Caused by Halothane Anesthesia and the Changes Taking Place in Plasma pH and Plasma CO_2 Content

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GENERAL anesthetic agents, because of their effects on the central nervous system, cause depression of pulmonary ventilation and subsequent changes in the plasma pH and the plasma CO_2 content of man and many other animals. Voluminous literature has accumulated on the clinical effects of general anesthetics, including halothane,* in man. Few records have appeared of the clinical use of halothane in domestic animals.[14, 15, 19]

The pharmacologic actions of halothane have been investigated with particular reference to its effects on the cardiovascular system.[7, 24]

The hypotension produced by halothane has been reported in clinical evaluations of this drug.[2, 4, 16, 23, 26] Some reports have appeared on renal and hepatic function after halothane anesthesia.[5, 18, 25] The effects of halothane on the respiratory system have been mentioned in various clinical assessments of the drug.[2, 4, 6, 17, 26] Devine *et al.*[12] have investigated its effects on respiratory rate, ventilation rate, and CO_2 output in man.

The effects of general anesthetics on respiration of cattle have not been studied fully. Volatile and gaseous anesthetics have been considered unsuitable for cattle because of the copious salivation and outpouring of bronchial secretions, with a risk of postanesthetic pneumonia.[27] Ether was reported to be ineffective as a general anesthetic for large animals,[8] although Henkels[16] described its use as a general anesthetic for horses. The successful use of volatile and gaseous anesthetics on adult cattle has been reported by Hall,[13] Hannson and Johannison,[10] and Fisher and Jennings.[11] Hall,[13] in a clinical evaluation of the effects of halothane on 4 cows, stated that respiratory depression and slowing of the pulse rate occurred. Hannson and Johannison[10] reported the successful use of a mixture of barbiturate, succinylcholine, and nitrous oxide as a general anesthetic for cattle. They observed slight changes in the blood pH and the partial pressure of CO_2 in the blood. Some of the changes they reported were not of respiratory origin since, on occasion, a rise in the partial pressure of CO_2 occurred with a rise in the plasma pH. As they also assisted ventilation by means of a pump, it was not possible to draw conclusions on the effects of the anesthetic mixture on respiration.

More clinical reports have appeared in veterinary literature on the use of nonvolatile general anesthetic agents in horses and cattle. Wright[27] reported an increase in pulse and respiratory rates of cattle during maintenance of general anesthesia with chloral hydrate. Henderson[14] has reported that the use of thiopental sodium causes a decrease in respiratory rate and an acceleration of the pulse of cattle. Parry[20] has reported on the use of *beta*-naphthoxyethanol* in horses, and Wright[27] reported that tachypnea and dyspnea were caused when this drug was used in cattle.

After the preliminary reports of the effectiveness of halothane as a general anesthetic agent,[26] this drug was made available for trial as a general anesthetic for large domestic animals. An apparatus was developed for the efficient administration of volatile and gaseous anesthetics to large animals,[10] and a clinical trial was then made of halothane as a general anesthetic agent for cattle and horses.[11]

In this report, studies were carried out on the effects of halothane anesthesia on pulmonary ventilation, plasma pH, and plasma CO_2 content of cattle mainly, but also of horses, sheep, and dogs.

METHODS AND APPARATUS

Apparatus.—Because of the high cost of halothane, it was necessary to administer the drug in a manner which gave maximum economy. A circle absorber of a size suitable for large domestic animals was constructed. The apparatus used is illustrated (fig. 1). Details of the use of this apparatus for anesthetiration of farm animals with different anesthetic agents has been previously reported.[21]

The same apparatus was used for anesthetization of dogs, sheep, and calves, but 1½-inch unidirectional valves were replaced with ¾-inch diameter unidirectional valves. Connections from

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* Bromochlorotrifluoroethane, manufactured as Flurothane by the Imperial Chemical Industries, Ltd., Great Britain, and available from Fort Dodge Laboratories, Fort Dodge, Iowa, as Halsan.

* Anavenol, manufactured by the Imperial Chemicals (Pharmaceuticals) Ltd.. Cheshire, England.

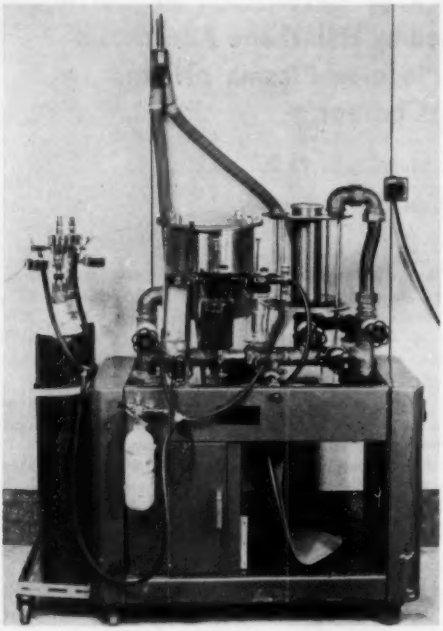

Fig. 1—Circle absorber anesthetic apparatus.

these smaller valves to the animal being anesthetized were standard, ¾-inch (internal diameter), nonkinking rubber tubing.

Methods.—Food and water were not withheld from any animal and they were sometimes anesthetized shortly after normal feeding. No preanesthetic drugs were used. A nylon catheter was introduced into the jugular veins of horses and cattle about 1 hour before anesthesia so that blood samples could be withdrawn easily before, during, and after anesthesia. Ventilation rates were measured in cattle and dogs for a control period of 10 minutes before anesthetization, using a Parkinson Cowan CD4 gas meter,* face masks of appropriate size, and unidirectional valves. Ventilation rates were measured only in 1 of 3 horses and 1 of 3 sheep because they became excited when a mask was applied to the face. A blood sample was taken from each animal before anesthetization, the plasma was separated, and the pH and total CO_2 were determined. Prior to anesthetization, some cattle were made to breathe pure oxygen for 10 minutes, during which time ventilation rate was recorded and blood samples were again taken for the determination of the plasma pH and the plasma CO_2 content.

Anesthetization was commenced in every case with the animal in the standing position. As anesthesia advanced, the animals were allowed to sink to the ground or, as with the majority of horses and cattle, a mechanically operated table was used, which was brought from the vertical to the horizontal position so that the animal was in lateral recumbency.

Two methods of induction were used. In the first, anesthesia was induced with a 10 per cent solution of thiopental sodium in saline solution. During this type of induction, it was not usual to record the ventilation rate. In the second method, halothane was used as the induction agent and, in some of the animals, the ventilation rate was recorded during induction by including a gas meter in the anesthetic circuit. In both methods, halothane was used to maintain anesthesia. When halothane was used for induction, a close-fitting face mask was applied to the animal and the circle absorber was connected to the face mask. The halothane was administered to the animal from a trichloroethylene vaporized* bottle by passing oxygen through the halothane in the vaporizer bottle before leading the oxygen into the anesthetic circuit.

Once general anesthesia had been induced, a cuffed endotracheal tube was introduced into the trachea. The circle absorber was then connected to the endotracheal tube and anesthetization was continued. Ventilation rates were measured during anesthesia by including the gas meter in the circuit between the expiratory valve and the rest of the circle absorber.

As soon as possible after induction, a blood

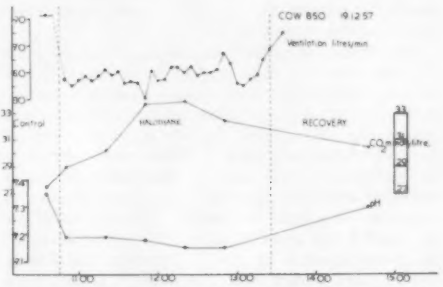

Fig. 2—Changes in ventilation rate, plasma CO_2 content, and plasma pH of cow 850/19/12 during halothane anesthesia.

sample was taken for the determination of the plasma pH and the plasma CO_2.

Once surgical anesthesia had been attained after connecting the circle absorber, the oxygen input was allowed to by-pass the halothane in the vaporizer. Halothane was added at intervals to maintain the desired plane of anesthesia, making up

* Parkinson & Cowan Industrial Products, Dobcross, Lancs., England.

* Imperial Chemical (Pharmaceuticals) Limited, Cheshire, England.

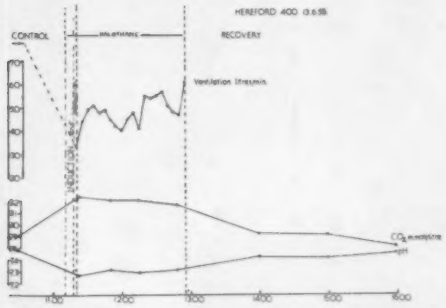

Fig. 3—Changes in ventilation rate, plasma CO_2 content, and plasma pH of bull 400/13/6 during halothane anesthesia.

for losses due to leakage from the circuit. The ventilation rate per minute was recorded continuously, and blood samples were taken at half-hour intervals for determination of the plasma pH and the plasma CO_2 content.

After the period of anesthetization, the circle absorber apparatus was disconnected. To guard against possible inhalation of saliva or food, the endotracheal tube was left *in situ* until the stage of recovery was reached where the subject would no longer tolerate the tube in the trachea. Ventilation rates were recorded to this point and on occasions afterwards. Blood samples were taken at intervals until the plasma pH had returned to the control value.

RESULTS

Cow 850/19/12.—After control values were identified, anesthesia was induced and maintained with halothane. Determinations were made during anesthesia. A blood sample was taken 60 minutes after the anesthetic circuit was disconnected. The changes which took place are illustrated (fig. 2).

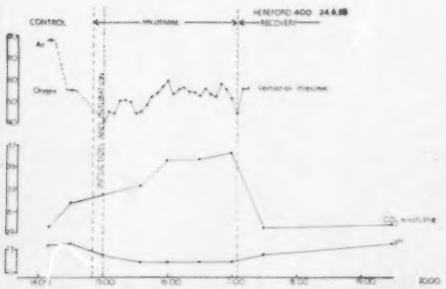

Fig. 4—Changes in ventilation rate, plasma CO_2 content, and plasma pH of bull 400/24/6 during halothane anesthesia.

A depression of pulmonary ventilation occurred and, at the same time, there was a fall in plasma pH and a rise in plasma CO_2. There was evidence of CO_2 retention and a low plasma pH 1 hour after removal from the anesthetic atmosphere.

Bull 400/13/6.—The procedures were the same as those described for cow 850/19/12.

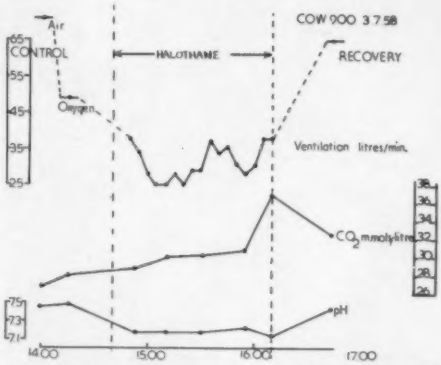

Fig. 5—Changes in ventilation rate, plasma CO_2 content, and plasma pH of cow 900/3/7 during halothane anesthesia.

The changes which took place are illustrated (fig. 3).

Again, a depression of ventilation occurred with a fall in plasma pH and a rise in plasma CO_2 content. During recovery at 60 minutes there was still some CO_2 retention.

Bull 400/24/6.—This animal was made to breathe pure oxygen for 10 minutes when unanesthetized. There was a depression of ventilation and a rise in plasma CO_2 content (fig. 4).

During anesthesia, induced and maintained with halothane, there was a depression of ventilation. Throughout the main part of anesthesia, however, the ventilation rate was not greatly different from that observed when the animal breathed pure oxygen. There was also CO_2 retention and a fall in plasma pH. The plasma pH and CO_2 content took 2 hours to return to the control levels.

Cow 900/3/7.—The procedures were the same as those described for bull 400/24/6. The changes which took place are illustrated (fig. 5).

Pre-anesthetic depression of ventilation occurred when the animal was breathing

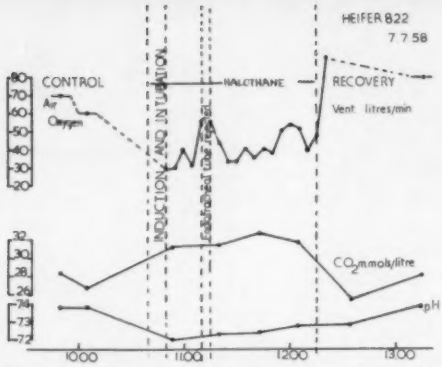

Fig. 6—Effects of halothane on the ventilation rate, plasma pH, and plasma CO_2 content of heifer 822/7/7.

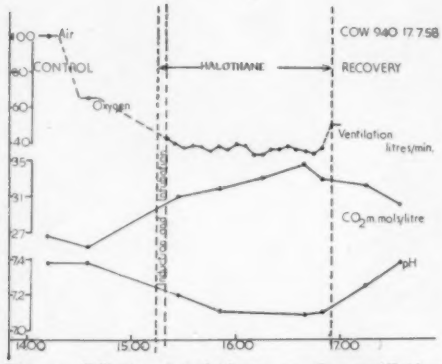

Fig. 7—Effects of halothane on the ventilation rate, plasma pH, and plasma CO_2 content of cow 940/17/7.

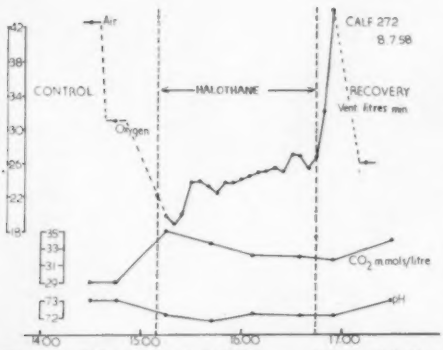

Fig. 8—Effects of halothane on the ventilation rate, plasma pH, and plasma CO_2 content of calf 272/8/7.

pure oxygen. During anesthesia, further depression of ventilation rate occurred. Plasma pH and plasma CO_2 both rose when the cow was breathing pure oxygen; during anesthesia, CO_2 retention was accompanied by a fall in plasma pH.

Plasma pH returned to the pre-anesthetic control value 30 minutes after removal from the anesthetic atmosphere, but there was still some retention of CO_2.

Heifer 822/7/7.—This 2-year-old heifer, subjected to the procedures described for bull 400/24/6, ejected the endotracheal tube in the middle of the experiment. Some respiratory depression occurred during the administration of pure oxygen, but the animal struggled violently, which may have made the depression less marked (fig. 6). The plasma CO_2 gave no evidence of retention during inhalation of pure oxygen. During anesthesia, depression of ventilation occurred with CO_2 retention and a fall in plasma pH. During recovery, some hyperventilation occurred. At the end of an hour, the plasma pH and plasma CO_2

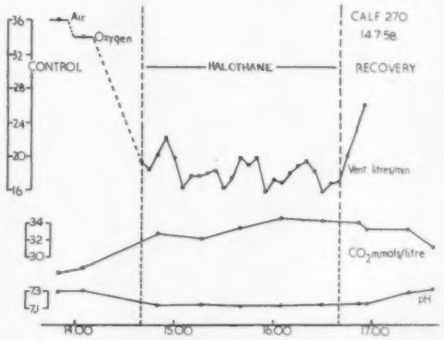

Fig. 9—Effects of halothane on the ventilation rate, plasma pH, and plasma CO_2 content of calf 270/14/7.

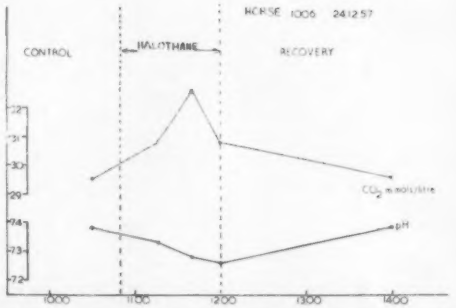

Fig. 10—Effects of halothane on plasma pH and plasma CO_2 content of horse 1006/24/12.

content had returned to pre-anesthetic control values.

Cow 940/17/7.—The procedures described for bull 400/24/6 were followed. The changes which took place are illustrated (fig. 7).

Inhalation of pure oxygen caused a depression of ventilation, but no marked changes were detected in the plasma pH or the plasma CO_2 content. Further depression of ventilation, with CO_2 retention and a fall in plasma pH, occurred during anesthesia. The plasma pH returned to normal 1 hour after removal of the cow from the halothane atmosphere, but there was still some CO_2 retention.

Calf 272/8/7.—The procedures described

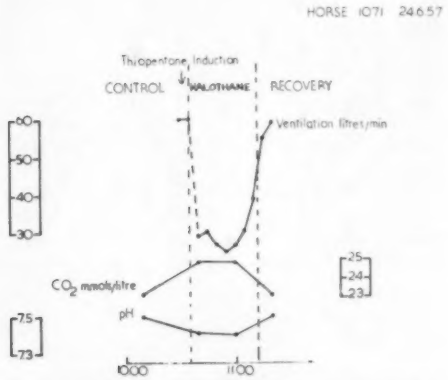

Fig. 11—Effects of halothane on the ventilation rate, plasma pH and plasma CO_2 content of horse 1071/24/6.

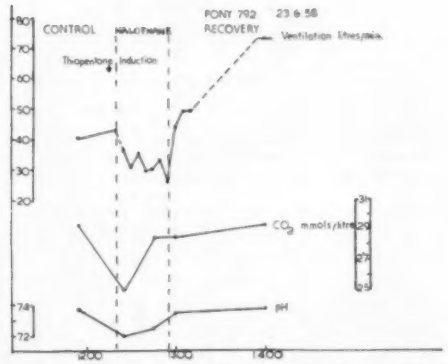

Fig. 12—Effects of halothane on ventilation rate, plasma pH, and plasma CO_2 content of pony 792/23/6.

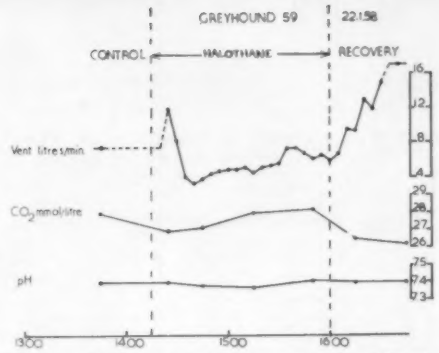

Fig. 13—Effects of halothane on the ventilation rate, plasma pH, and plasma CO_2 content of greyhound 59/22/1.

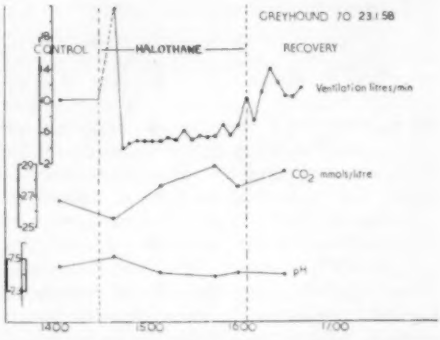

Fig. 14—Effects of halothane on the ventilation rate, plasma pH, and plasma CO_2 content of greyhound 70/23/1.

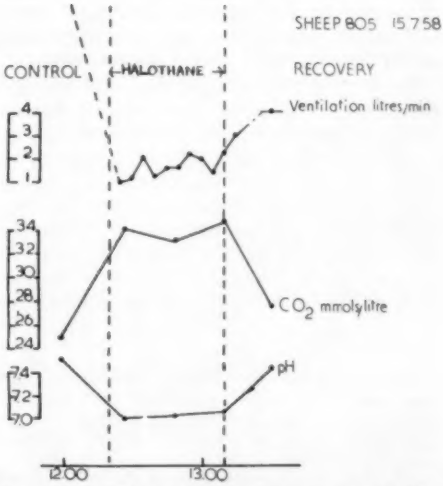

Fig. 15—Effects of halothane on the ventilation rate, plasma pH, and plasma CO_2 content of sheep 805/15/7.

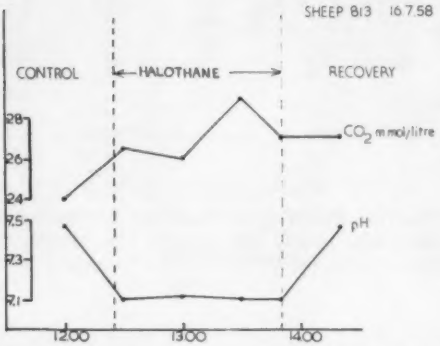

Fig. 16—Effects of halothane on plasma pH and plasma CO_2 content of sheep 813/16/7.

for bull 400/27/6 were followed. In this calf (10 months old), the changes were similar to those in adult cows. There was preanesthetic depression of ventilation during inhalation of pure oxygen, but changes were not detected in the plasma pH and the plasma CO_2 content. Further depression of ventilation occurred during anesthesia but, although this was marked immediately after intubation, over the whole period of anesthesia the depression was not great. Changes of plasma pH and plasma CO_2 reflected the ventilation changes (fig. 8). During recovery, the plasma pH returned to the control value after half an hour, although after a short period of hyperventilation the ventilation rate was depressed and there was still some CO_2 retention.

Calf 270/14/7.—The procedures described for bull 400/24/6 were followed. The changes are illustrated (fig. 9).

This calf had only slight depression of ventilation and a slight rise in plasma CO_2 when inhaling pure oxygen. During anesthesia, a marked depression of ventilation occurred with CO_2 retention and fall in plasma pH. During recovery, plasma pH had returned to near control values, but after 45 minutes there was still some CO_2 retention.

Horse 1006.—In this horse, halothane was used for both induction and maintenance of anesthesia. The ventilation rate was not measured.

During anesthesia, the rise in plasma CO_2 and the fall in plasma pH indicated a depression of ventilation (fig. 10). One hour after removal of the anesthetic atmosphere, plasma pH and plasma CO_2 had returned to the control values.

Horse 1071.—Induction and intubation were carried out with thiopentone sodium as the anesthetic agent. Ventilation was recorded after induction. The changes which took place are illustrated (fig. 11).

Pony 792.—The procedures used for horse 1071 were followed. In this pony it was possible to follow the ventilation rate. Following induction and intubation with thiopentone sodium, some respiratory stimulation occurred so that the mean value for the ventilation rate for the 5 minutes after halothane had been given to the pony did not indicate respiratory depression (fig. 12). The plasma CO_2 at this point indicated hyperventilation, but the plasma pH had also fallen and may have indicated some degree of metabolic acidosis as seen by Bunker *et al.*[5] during ether anesthesia in dogs. Beyond this point, some respiratory depression occurred with a rise in plasma CO_2 content. During the recovery period, after 5 minutes, plasma pH and plasma CO_2 were nearly back at the control values. One hour later they had returned to the control value. One hour after anesthesia, when the ventilation rate was measured, some hyperventilation was observed.

Greyhound 71.—Anesthesia was induced by means of halothane. The changes which took place are summarized (table 1).

TABLE 1—Effects of Halothane on the Ventilation Rate, Plasma pH, and Plasma CO_2 Content in Greyhound 71

	Pre-anesthesia	Anesthesia (time in min.)			Recovery*
		5	30	90	
Plasma pH	7.40	7.36	7.36	7.38	7.39
Plasma (mM CO_2/liter)	29.00	30.30	30.50	30.30	30.30
Ventilation (liters/min.)	7.60	4.60	5.00	5.00	12.50

* Twenty-five minutes after the dog was removed from the anesthetic atmosphere.

The introduction of the endotracheal tube caused a marked stimulation of respiration which was not measured. Once the respiratory stimulation had ceased, the ventilation rate was again recorded. A depression of ventilation occurred during halothane anesthesia, but marked changes were not detected in plasma pH and plasma CO_2. During recovery, marked hyperventilation occurred.

Greyhound 59.—This dog also was given halothane for induction of anesthesia. As in the previous greyhound, intubation caused stimulation of respiration and hyperventilation (fig. 13). Respiratory depression then occurred. The changes in plasma pH were small during anesthesia. The plasma CO_2 fell as a consequence of the hyperventilation and then gradually rose again. During the recovery period, the plasma pH remained stationary but the plasma CO_2 fell, since ventilation rate was above the control rate.

Greyhound 70.—Induction was by means of halothane. The changes which took place are illustrated (fig. 14).

Intubation again caused a marked hyperventilation which lowered the plasma CO_2 content and caused the plasma pH to rise. Following hyperventilation, respiratory depression took place, causing slight CO_2 retention and a fall in plasma pH. Twenty-five minutes after the dog had been removed from the halothane atmosphere, the pulmonary ventilation was similar to the control value. However, there was still some CO_2 retention and a plasma pH below the control value.

Sheep 805.—Induction of anesthesia was by halothane. Ventilation rates were recorded (fig. 15).

In this experiment, excitement caused hyperventilation before anesthesia. Marked respiratory depression occurred with striking changes in both the plasma pH and the plasma CO_2 content. Once the sheep was removed from the anesthetic atmosphere, both the plasma pH and the plasma CO_2 returned to near the control values.

Sheep 813.—Halothane was used to induce anesthesia. The changes of ventilation in this sheep were not recorded.

Marked changes in the plasma pH and the plasma CO_2 content occurred during halothane anesthesia (fig. 16). During recovery, the plasma pH returned to the control pre-anesthetic value before the plasma CO_2 did.

Sheep 823.—Anesthesia was induced by means of halothane. Ventilation rate changes in this sheep were not recorded.

Marked changes occurred in both plasma pH and plasma CO_2 during halothane anesthesia, but both plasma pH and plasma CO_2 had returned to the control values 40 minutes after removal from the halothane atmosphere (table 2).

TABLE 2—The Effects of Halothane on Plasma pH and Plasma CO_2 Content in Sheep 823

	Pre-anesthesia	Anesthesia (time in min.) 5	30	60	Recovery*
Plasma pH	7.54	7.13	6.98	7.44	7.55
Plasma (mM CO_2/liter)	24.00	31.40	34.30	25.40	23.00

* Forty minutes after sheep was removed from the anesthetic atmosphere.

DISCUSSION

In each of the species studied, halothane anesthesia produced respiratory depression and a fall in the ventilation rate. The greatest respiratory depression occurred in sheep, in which halothane often produced apnea so that artificial respiration had to be commenced. Part of the respiratory depression which occurred may be explained by the concomitant administration of pure oxygen, since it was shown that unanesthetized cattle had lower ventilation rates when breathing pure oxygen. Changes in the plasma CO_2 content occurred in all species studied and were consistent with the changes in pulmonary ventilation. A lowering of the pulmonary ventilation caused CO_2 retention and a raising of the plasma CO_2 content, while an increase in pulmonary ventilation caused a fall in the plasma CO_2 content.

It was observed that in prolonged periods of anesthesia there was often a stabilization of the plasma CO_2 content at a higher concentration than normal and, towards the end of anesthesia, a tendency for the CO_2 concentration to fall. These results indicate that there was an efficient soda-lime absorption of CO_2 in the anesthetic apparatus used, since a continuous build-up of CO_2 did not occur. The stabilization which occurred may be explained in the light of the work of Quastel,[22] who showed that, during barbiturate anesthesia, tissue metabolism was decreased. In that case, CO_2 production would be decreased also. The decreased CO_2 production could then be handled by the decreased ventilation.

The changes taking place in the plasma pH during halothane anesthesia are consistent with CO_2 retention and a respiratory acidosis. Recovery from halothane anesthesia was rapid and, during recovery, it was observed that the plasma pH often returned to the pre-anesthetic value before the plasma CO_2 content returned to the

control value. It was inferred from this that some extra buffering capacity or buffer-regulating mechanism which had previously been inhibited by the anesthesia came into action during recovery. This could indicate an interference with renal regulation as has been shown to occur with barbiturate anesthesia,[2] but this possibility requires confirmation.

SUMMARY

The effects of halothane anesthesia on the plasma pH, the plasma CO_2 content, and the ventilation rate have been studied principally in cattle, but also in horses, sheep, and dogs. The anesthetic caused a depression of the ventilation rate in all species studied and, concomitant to this, a respiratory acidosis occurred. Removal of the animal from the anesthetic atmosphere produced rapid return of ventilation rate to that of preanesthetic levels as a consequence of a recovery from the respiratory acidosis.

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SUMMARIO IN INTERLINGUA

Observationes Concernente le Disturbationes del Respiration Causate per Anesthesia a Halothano in Bestial, Cavallos, Oves, e Canes e le Alterationes Occurrente Concomitantemente in le pH del Plasma e in le Contento Plasmatic de CO_2

Le effectos de anesthesia per halothano super le pH del plasma, le contento plasmatic de CO_2, e le ventilation esseva studiate principalmente in bestial sed etiam in cavallos, oves, e canes. Le anesthetico produceva un depression del ventilation in omne le species studiate e—concomitantemente—le occurrentia de un acidosis respiratori. Le remotion del animales ab le atmosphera del anesthetico resultava in le rapide retorno del ventilation al nivellos preanesthetic e—per consequente—un restablimento ab le acidosis respiratori.

Biological Assays of Extracts of Alfalfa Samples from Bloat-Producing Pastures

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RECENT STUDIES to develop and test the validity of theories explaining the cause of acute bloat in ruminants have not promoted agreement among contemporary workers.[3, 18, 20, 24, 26] Although evidence obtained by many investigators[1, 5, 6, 12, 19, 27, 33] favors an explanation by physical factors of forage and ingesta, others[10, 15–17, 23, 25, 29, 30, 32] have presented evidence that chemical factors in ingested legumes may contribute to the cause of acute bloat. Many toxic or physiologically active factors have been suspected. However, there is little evidence that bloat-producing legumes contain a factor in higher concentration than nonbloat-producing legumes.

The present study was made in an attempt to find methods for assay of materials in legumes which may be in part responsible for the development of the symptoms of acute bloat. Methods are described here for a systematic extraction procedure applied to legume samples from known bloat-producing and known nonbloat-producing pastures. Methods for chemical and biological assay of such extracts are given.

METHODS

SAMPLE COLLECTION AND EXTRACTION

Alfalfa samples were collected from 2 general sources (the Soils Farm at the Rosemount Research Center, University of Minnesota, and privately owned farms surrounding the Minneapolis-St. Paul area). During the pasture seasons of 1957, 1958, and 1959, 20 samples were collected under a variety of conditions. A limited record was kept of grazing management, bloat incidence, percentage alfalfa, plot size, and soil fertilization. Of the samples, 11 were obtained the day bloat occurred on the pastures; 3 were collected from pastures grazed by cattle showing no symptoms of bloat and 6 from ungrazed pastures.

Harvesting of alfalfa samples consisted of cutting off the top half of the plants with either a hand scythe or a small hand-operated power mower. The alfalfa tops were placed directly on slabs of dry ice 1 inch thick, in polyethylene bags.* The bags were transported immediately to a large freezer maintained at −16 C.

The leaves were stripped from the stems while still frozen. The stems were not used. Frozen leaves (1.5-kg. amounts) were placed in a stainless steel blender** and enough distilled water (1.5 to 2.0 liters) was added to allow homogenization. The following procedures were used in the initial studies:

1) The homogenate was subjected to steam distillation, collecting from 3 to 9 fractions of the distillate. The remaining homogenate was filtered through cheesecloth and the solids discarded.

2) To the filtrate (pH 5.5 to 6.0) placed in a 6-liter separatory funnel were added three 1.5-liter portions of chloroform. The resulting chloroform extracts were combined and the chloroform was removed by vacuum distillation at 45 C. The residue was extracted first with 10 ml. of 0.1 N HCl, then with 10 ml. of 0.1 N NaOH using 5 ml. of distilled water to rinse the flask after each treatment of the residue. The HCl-soluble material was labeled "nonalkaloid in HCl." The NaOH-soluble material was labeled "nonalkaloid in NaOH."

3) The pH of the aqueous phase was then adjusted to 9.5 with Na_2CO_3. The chloroform extractions were repeated as above. The HCl- and NaOH-soluble materials were labeled, "alkaloid in HCl," and "alkaloid in NaOH," respectively.

4) The remaining aqueous phase was then centrifuged and the supernate was concentrated by vacuum distillation at 55 C. until the volume was

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* The polyethylene bags (25 by 36 inches, 0.003 inch thick) were supplied by Shelly Inc., Farmington, Minn.

** Model CB 3, Waring Blendor, Waring Products Corp., New York, N.Y.

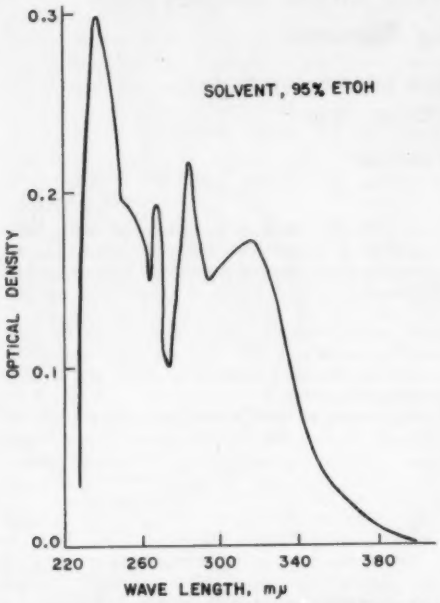

Fig. 1—Ultraviolet absorption spectrum of Soxhlet extract of dried leaves, alfalfa sample No. 19 (St. Paul campus experimental plot X28, 7-21-59), extract diluted with 95 per cent ethanol.

400 ml. This fraction was labeled "aqueous residue, concentrated."

5) A 100-ml. sample of the aqueous residue concentrate was placed in a cellulose dialyzer tubing. The filled tubing was placed in 1 liter of distilled water in a refrigerator (8 C.). The water was changed each day for 4 days. The first 24-hour diffusate and the nondiffusible portion were saved for bioassay.

6) The first 24-hour diffusates were concentrated by vacuum distillation to a 100-ml. volume. The resulting fraction was divided into 2 equal volumes. One half was allowed to pass through a column of Dowex 50W-X 1 cation exchange resin.

All of the extracts were refrigerated until use. Immediately before the biologic tests were to be made, the pH of each extract was adjusted to 6.5 to 7.5 with either HCl or Na_2CO_3.

Later in the studies, modifications were introduced into the extraction procedure. The first modification was as follows: frozen leaves were homogenized using 0.1 N NaOH instead of distilled water. Steam distillation was omitted. The pH of the homogenate filtered through cheesecloth was adjusted to 5.0 with 6.0 N HCl. Three 1.5-liter chloroform extractions were made for each 1.5 kg. of frozen leaves. The chloroform was removed by vacuum distillation at 45 C. until the volume was 50 ml. Then 40 ml. of 5 per cent $NaHCO_3$ were added and the remaining chloroform removed by vacuum distillation. The $NaHCO_3$ soluble material was filtered through No. 41 Whatman paper and then a clarifying asbestos filter. The pH of the filtrate was adjusted to 2.0 with 6.0 N HCl and extracted 3 times with ether. Following the removal of ether, the ether residue was dissolved in 1.0 N NaOH. The pH of the solution was adjusted to 7.4 with 1.0 N HCl and refrigerated until bioassay.

The second modification was as follows: ground dried leaves were extracted with chloroform in a 500-ml. Soxhlet extraction chamber, continuing extraction until no more colored material was removed. The resulting chloroform extract was treated as in the first modification above.

BIOLOGIC ASSAY

Isolated Rabbit Ileum.—This technique, patterned after the method described by Gaddum,[14] was used most extensively. Six 3-cm. sections of ileum were removed from a rabbit and placed in Ringer-Tyrode solution at room temperature until use. The muscle-warming tube (capacity = 11.5 ml.) was made from a Pyrex test tube. Through a rubber stopper at the bottom of the tube, Ringer-Tyrode solution at 37 C. flowed in at a rate of 11.5 ml. per minute. Excess Ringer-Tyrode solution was allowed to spill over into the large water bath surrounding the tube. Oxygen bubbled in at a rate just fast enough to give adequate mixing of the muscle bath. The intestinal segment was fixed by a hook and thread to the rubber stopper and a lever which wrote on moving smoked paper.

Rabbit uterus was subjected to study in some of the later work. It was obtained at the same time as ileum and tested in the same manner.

Turtle Vagus.—Extracts were applied directly with a small dropper to the exposed cervical vagus nerves of singly pithed turtles. A thread attached to a lever which wrote on a moving smoked paper recorded ventricular beats of the exposed heart. Normal nerve conduction was determined by stimulating the peripheral end of the vagus with a square wave stimulator* and observing cardiac inhibition. Each period of stimulation lasted 10 seconds or less. Various stimuli were tried on a given turtle nerve until at least 5 consecutive stimulations produced cardiac inhibition. Extracts were applied to a nerve for 5 minutes or until stimulation no longer produced cardiac inhibition. The nerve was then flushed with Ringer-Locke solution to remove residual extract. Stimulations were done at regular periods between and during extract application. In this way, a constant check was made of the functional status of the nerve.

Mouse Injections.—Young male mice weighing about 20 Gm. were injected intraperitoneally with 1 ml. of each extract. Mice were observed every 5 minutes for 3 hours after injection, then every 12 hours for 5 days.

Rabbit Cornea.—Extracts were applied with a dropper directly on the cornea. One eye served as

* Model 751, American Electronics Laboratories, Inc., Philadelphia, Pa.

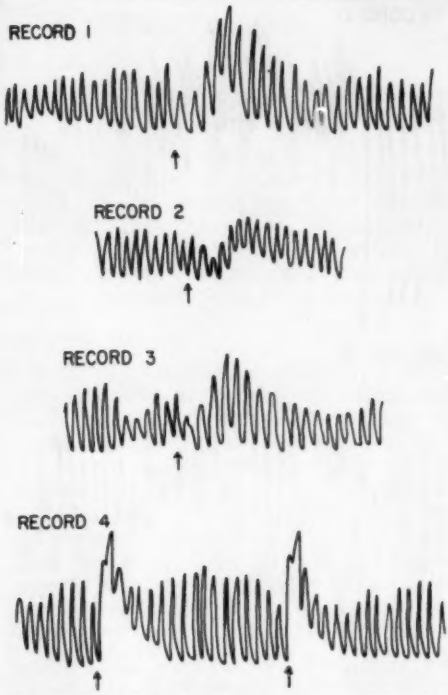

Fig. 2—Record 1—Tracing of response of isolated rabbit ileum to 2 ml. of distilled water at 4 C. Time = 4.0 minutes.*
Record 2—Tracing of response of isolated rabbit ileum to 2 ml. of distilled water at 38 C. Time = 2.3 minutes.
Record 3—Tracing of response of isolated rabbit ileum to 2 ml. of Ringer-Tyrode solution at 4 C. Time = 2.8 minutes.
Record 4—Tracing of response of isolated rabbit ileum to 0.0005 mg. acetylcholine chloride. Time = 4.0 minutes.
***"Time" refers to the duration of the entire picture of the tracing in each of the records 1-19c.**

a control and isotonic saline solution was applied to it each time extract was applied to the other eye. The pupillary response to light was checked in some cases. In others, the corneal reflex was tested by applying a fine horsehair bristle to the cornea.

Cat Blood Pressure.—Extracts were injected intravenously in anesthetized cats which had been previously prepared for recording carotid arterial blood pressure with a recording mercury manometer.

Bovine Injections.—Five cows with rumen fistulas were injected intravenously with selected extracts. Symptoms after injection were noted. During some trials, recordings were made of the electrocardiogram and of the pressure changes in parts of the forestomachs and cardia. The pressure recording system has been described previously.[8] Only precordial leads of the ECG were recorded.[28]

Results

Sample Collection, Extraction, and Analysis.—Bloating occurred during all months of the usual pasture season (June to September), during all stages of alfalfa growth, and during various times of day and night. Bloating occurred on both fertilized and nonfertilized plots. No special attempts were made to relate soil management to bloat incidence. However, in those 8 cases where a history of soil management was available and bloat occurred, 7 samples were from nonfertilized pastures. When adequate grass was available to grazing cattle, bloating did not occur because cattle selected grass in preference to alfalfa.

Individual animal susceptibility to bloat from year to year was not determined because steers at Rosemount were slaughtered at the end of each pasture season. No records could be made of individual animals showing symptoms of bloat on privately owned farms. At Rosemount in 1957, bloating occurred repeatedly and a record was

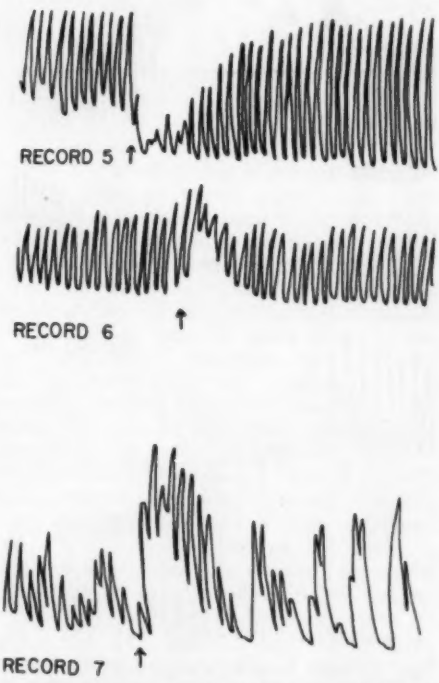

Fig. 3—Record 5—Tracing of response of isolated rabbit ileum to 0.001 mg. epinephrine hydrochloride. Time = 4 minutes.
Record 6—Tracing of response of isolated rabbit ileum to 2 ml. KCl (50 mEq./liter of Ringer-Tyrode solution). Time = 4 minutes.
Record 7—Tracing of response of isolated rabbit ileum to 1 ml. 1 N HCl with pH adjusted to 7.0 with a saturated soultion of Na_2CO_3, approximately 5 times isotonic NaCl solution. Time = 4 minutes.

kept of each case. During the 12 days when bloating was recorded, 1 steer bloated on 11 and 1 on 6 of these days. Of the remaining 16 of the 37 steers which bloated that year, each bloated on 1 or 2 days only. The 3 bloated steers that died at Rosemount during 1957 and 1958 were discovered dead in the morning.

Ultraviolet (UV) absorption curves (determined with the Beckman DU spectrophotometer) of the 4 fractions obtained from each alfalfa sample with chloroform extraction were studied in 5 samples from bloat-producing and 3 samples from nonbloat-producing pastures. Although each fraction contained many substances, the nonalkaloid in NaOH fractions which consistently produced biologic responses contained 5 to 10 times more material with UV absorption at 270 and 280 mμ than any other fraction. Further support for the relation between absorption at these wave lengths and biologic activity came from study of a known biologically active fraction which lost activity when shaken with charcoal. Comparison of optical densities of before and after treatments showed that after charcoal treatment one fifth of the material absorbing at 270 and 280 mμ remained.

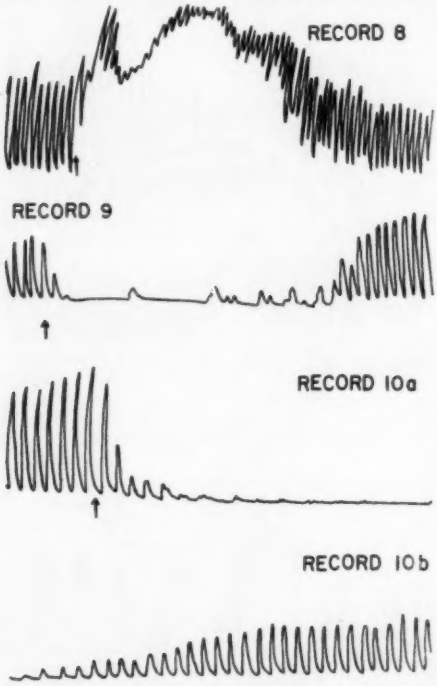

Fig. 4—Record 8—Tracing of response of isolated rabbit ileum to 1 ml. saturated Na_2CO_3 solution at pH 11.3. Time = 4 minutes.
Record 9—Tracing of response of isolated rabbit ileum to 2 ml. Ringer-Tyrode solution containing traces of chloroform, prepared by shaking equal volumes of Ringer-Tyrode solution and chloroform at 38 C. Time = 4 minutes.
Records 10a and 10b—Tracing of response of isolated rabbit ileum to 2 ml. of nonalkaloid in NaOH extract of alfalfa sample No. 19 (St. Paul campus experimental plot X28, 7-21-59). Time = 8 minutes.

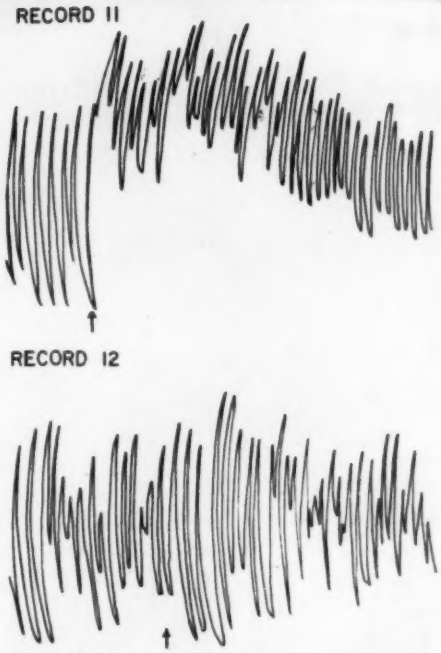

Fig. 5—Record 11—Tracing of response of isolated rabbit ileum to 1 ml. aqueous residue, concentrated, alfalfa sample No. 3 (Rosemount, pasture E-2, 7-18-57). Time = 4 minutes.
Record 12—Tracing of response of isolated rabbit ileum to 2 ml. nondiffusible extract, alfalfa sample No. 3 (Rosemount, pasture E-2, 7-18-57). Time = 4 minutes.

The absorption curve of an extract of dry leaves prepared by Soxhlet extraction is shown (fig. 1). Absorption peaks were approximately the same as those of extracts prepared from frozen leaves. In this fraction, peaks appeared at 237, 269, 284, and 315 mμ. No differences could be found between the concentration of absorbing material in nonalkaloid in NaOH fractions between bloat-producing and nonbloat-producing pasture samples.

Sodium and potassium concentrations determined by use of the Beckman DU flame photometer on fractions of the aqueous residues are listed (table 1). Both sodium and potassium concentrations (expressed in

mEq./liter) were high in first 24-hour diffusates and low in fourth 24-hour diffusates and nondiffusible fractions. It should be noted that the sodium concentrations would be influenced by the addition of Na_2CO_3 to the aqueous phase prior to the second series of chloroform extractions.

BIOLOGIC ASSAYS

Isolated Rabbit Ileum.—Sections of ileum were required from each of 53 rabbits. Each extract was tested an average of 3 times. Trials of comparable extracts from sample to sample showed similar results qualitatively.

To test the sensitivity of ileum in the apparatus, several solutions were tried. Records 1 and 2 (fig. 2) show typical responses of ileum to 2 ml. of distilled water at 2 temperatures, 4 and 38 C. The time interval for record 1 is 4 minutes. All records, 1 to 14, were made at the same kymograph speed. Water only at muscle bath temperature still produced a temporary reduction in tonus and contraction amplitude, followed by a rise in tonus. A balanced electrolyte solution, record 3 (fig. 2) at 4 C., produced a decrease in tonus and amplitude followed by a rise in tonus and amplitude. Electrolyte balance and temperature of test solution both had to be similar to that of the muscle bath or alterations in motility pattern appeared.

It was thought that the continuous flow of new Ringer-Tyrode solution into the muscle-warming tube would make the preparation insensitive to autonomically active substances, rendering the procedure useless. Records 4 and 5 (fig. 2, 3) show typical tracings of the responses to acetylcholine and epinephrine. The preparations were capable of responding (in a manner similar to that seen in records 4 and 5) to 0.00025 mg. of acetylcholine and 0.0005 mg. of epinephrine, indicating that, although duration of response was short, under these conditions sensitivity was still great. Constant infusion of new Ringer-Tyrode solution provided for rapid removal of test material without interfering with motility patterns. This allowed testing of several materials on each ileal segment, an essential feature of bioassays used. As many as 30 extracts were tested on ileal segments from 1 rabbit.

Each ileal segment contracted with easily recordable amplitude and regularity for about 2 hours. When amplitude and regularity of spontaneous contractions decreased, new segments were used. Those segments stored at room temperature in Ringer-Tyrode solution were capable of good motility patterns for up to about 6 hours. After this time, spontaneous motility became erratic. The method of killing the rabbit did not appear to be important to good spontaneous motility. Stretching and handling tissues during removal and washing to remove ingesta seemed to be most harmful to segments.

TABLE 1—Sodium and Potassium Concentrations in Aqueous Residue Fractions

Fraction	No. pasture samples examined	mEq./liter	
		Sodium	Potassium
1st 24-hr. diffusate	4	412–1037	96–150
1st 24-hr. diffusate	4	.....	115–160
4th 24-hr. diffusate	4	<1	<0.4
Nondiffusible	5	7–20	0.7–1.3
1st 24-hr. diffusate through cation exchange resin column	3	0.4 to 1.5	.....

Further support for importance of electrolyte balance in test solutions is illustrated in records 6, 7, and 8 (fig. 3, 4). In record 6, the response of ileum to 2 ml. of KCl solution (50 mEq./liter) is shown. The first 24-hour diffusates contained as much as 160 mEq./liter in those fractions tested. An increase in tonus and decrease in amplitude of contraction was seen with KCl solutions and with aqueous residues and first 24-hour diffusates. Record 7 illustrates the effect of high NaCl concentration resulting from using 1.0 N HCl as a solvent for residues. The resulting solution of NaCl which is formed by a solution of 1.0 N HCl with pH adjusted to 7.0 with saturated Na_2CO_3 (c. 20 Gm./100 cc. at 22 C.) is about 5 times isotonic and would appear to be unsuitable for rabbit ileum testing. Use of 0.1 N HCl as a solvent results in about one half isotonic solution of NaCl which did not influence ileal motility pattern. Record 8 shows effects of saturated Na_2CO_3 solution at pH 11.3. These effects were seen with other solutions of high pH also. Record 9 (fig. 4) illustrates effects of the presence of chloroform in test solutions. Equal volumes of chloroform and Ringer-Tyrode solution were shaken, allowed to separate, and the Ringer-Tyrode solution at

38 C. was removed from the top layer with syringe and needle. The inhibitory response seen in this record is similar to that seen in records 10a and 10b (fig. 4) with nonalkaloid in NaOH extract. Alfalfa extracts, however, did not contain chloroform in inhibitory concentrations. Following vacuum distillation for chloroform removal, no other chloroform extracts showed inhibition and yet could have had as much chloroform present as the type shown in records 10a and 10b.

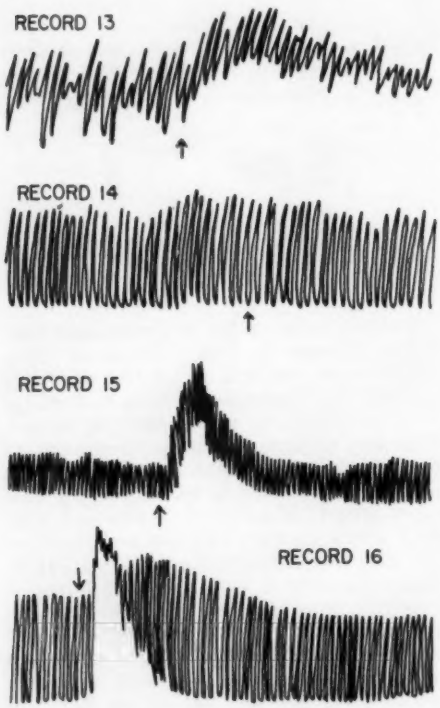

Fig. 6—Record 13—Tracing of response of isolated rabbit ileum to 2 ml. of first 24-hour diffusate, alfalfa sample No. 3 (Rosemount, pasture E-2, 7-18-57). Time = 4 minutes.

Record 14—Tracing of response of isolated rabbit ileum to 5 ml. of first 24-hour diffusate passed through cation exchange resin, alfalfa sample No. 3 (Rosemount, pasture E-2, 7-18-57). Time = 4 minutes.

Record 15—Tracing of response of isolated rabbit ileum to 0.1 ml. of pooled rumen liquid from cow No. 1923, pH adjusted to 7.4. Time = 10 minutes.

Record 16—Tracing of response of isolated rabbit ileum to 1 ml. of pressed juice from alfalfa sample No. 8 (Rosemount, pasture A-2, 9-6-57), pH 6.9 (not adjusted). Time = 10 minutes.

Presence of inhibitory material was shown in nonalkaloid in NaOH extract of 10 alfalfa samples, 6 of which were from bloat-producing pastures. No difference could be detected between concentrations of inhibitory material from bloat-producing and nonbloat-producing pastures.

Records 11–14 (fig. 5, 6) illustrate typical responses of ileum to fractions of aqueous residue. Aqueous residue and first 24-hour diffusate both caused a rise in tonus. Nondiffusible fractions and first 24-hour diffusate passed through a column of cation exchange resin produced no detectable alteration in motility pattern as seen in records 12 and 14.

Records 15 and 16 (fig. 6) show effects of crude rumen liquid and alfalfa juice, respectively. Each record represents 10 minutes' time. Rumen liquid samples from 6 fistulated cows were tested. Samples were prepared by first taking 12 samples from various parts of the rumen and reticulum and pooling, filtering through gauze, centrifuging at low speed, filtering the supernate through No. 41 Whatman paper, and adjusting the pH to 7.4. All 6 pooled samples gave the same response when tested on ileum.

Alfalfa juices were prepared from 17 samples by removing leaves from the stems, squeezing the juice out of the leaves, centrifuging, and filtering the supernate through No. 41 Whatman paper. The pH of each was determined (5.6 to 7.0) but no adjustment was made. Thirteen of 17 produced a response similar to that seen in record 16. The remaining 4 produced a brief period of decreased amplitude of contraction followed by a longer period (4 minutes) of increased amplitude of contraction.

By ligating the bottom end of the ileal segment and placing inhibitory extract inside the lumen so that none came in contact with the serosal surface, it was shown with 2 segments from 2 rabbits that inhibition occurred by extract contact with the serosal surface.

A series of experiments designed to determine some of the chemical properties of inhibitory material in the nonalkaloid in NaOH fraction by checking following each chemical treatment with rabbit ileum test showed the following properties of the material: (1) soluble in chloroform, 0.1 N NaOH, 5 per cent $NaHCO_3$, petroleum ether, methanol, ethanol, and diethyl ether;

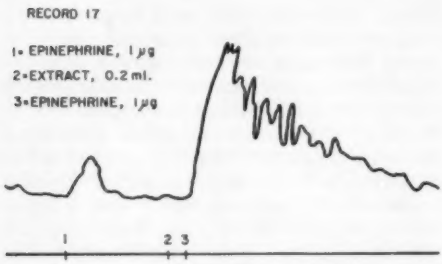

Fig. 7—Record 17—Tracing of isolated rabbit uterus, epinephrine response and enhancement of epinephrine response by nonalkaloid in NaOH extract, alfalfa sample No. 19 (St. Paul campus experimental plot X28, 7-21-59). Time =9.8 minutes. Notice that 0.2 ml. of extract alone does not affect uterus but enhances response to 1 μg. of epinephrine hydrochloride.

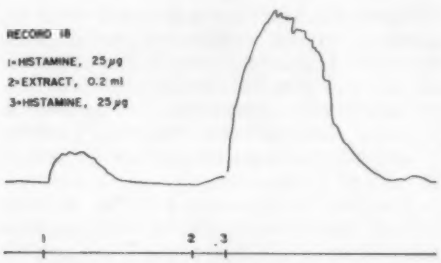

Fig. 8—Record 18—Tracing of isolated rabbit uterus, histamine response, and enhancement of histamine response by nonalkaloid in NaOH extract, alfalfa sample No. 19 (St. Paul campus experimental plot X28, 7-21-59). Time = 10.8 minutes. Notice that 0.2 ml. of extract alone does not affect uterus but enhances response to 25 μg. of histamine dihydrochloride.

(2) insoluble at or below pH 5 and in distilled water; (3) stable for 60 minutes, unstable for 140 minutes in a boiling water bath when in 0.1 N NaOH; (4) adsorbed by charcoal; (5) not eluted from charcoal by 0.1 N NaOH or chloroform; (6) strongly adsorbed or altered by Al_2O_3*; (7) precipitable as a green oil from a methanol-water mixture; (8) yellow-colored at an alkaline pH.

Dried leaf extract was found to contain inhibitory material also.

Steam distillates made isotonic with addition of NaCl did not alter ileal motility.

Nonalkaloid in NaOH extract did not affect uterine segments when introduced into the muscle-warming bath. However, as shown in records 17 and 18 (fig. 7, 8), the extract markedly enhanced the response of rabbit uterus to epinephrine and histamine. Record 17 represents 9.8 minutes; record 18, 10.8 minutes.

Turtle Vagus.—Twenty turtles were used for this experiment. Each fraction was tested from 1 to 3 times. Comparable fractions between alfalfa samples gave the same response.

Distilled water applied to the vagus for 15 minutes between electrode and heart did not block conduction. Since all solutions were maintained at room temperature, no temperature adjustment was required.

Aqueous residue, first 24-hour diffusate, and nonalkaloid in NaOH fractions consistently blocked vagus conduction. The time

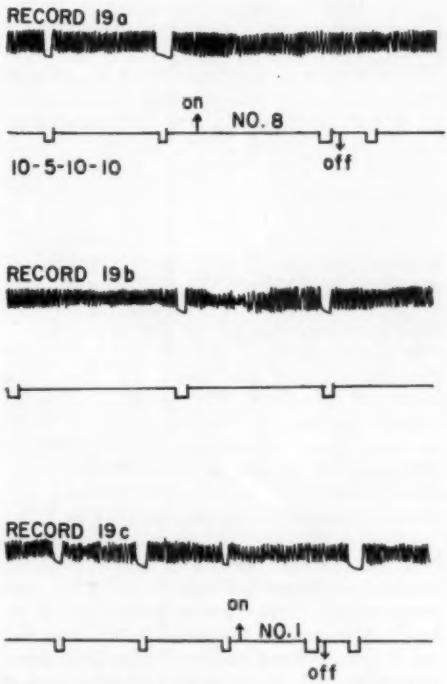

Fig. 9—Records 19a, b, and c—Tracing of turtle vagus inhibition by nonalkaloid in NaOH extract from 2 alfalfa samples (No. 8, Rosemount, pasture A-2, 9-6-57 and No. 1, Rosemount, pasture E-1, 6-27-57). Top tracing in each record is ventricular beat and bottom tracing indicates when stimulator is on or off. Time = 12 minutes. Vagus stimulation is indicated by a signal magnet deflection and is of duration equal to the depression shown.

* Alcoa activated alumina, Grade F-20, Aluminum Co. of America, East St. Louis, Ill.

required for blocking conduction after beginning extract application varied from less than 1 minute to 5 minutes. Aqueous residue and first 24-hour diffusate fractions usually blocked conduction in less than 30 seconds. The time required for recovery after initiation of washing procedure varied from 1 to 10 minutes.

Records 19a, 19b, and 19c (fig. 9) show typical responses of vagus nerve to nonalkaloid in NaOH fractions. The 3 records constitute a continuous recording of ventricular beats and turtle vagus stimulation over a 12-minute period. Vagal stimulation is indicated by depression of stylus and is of duration equal to depression shown. Application of extract is indicated by the arrow pointing up, and washing-off extract is indicated by the arrow pointing down. Stimulator settings are indicated in the lower left-hand corner of record 19a. Vagus nerve conduction is indicated by stopping of the heart during stimulation. Although it is not shown on these records, normal nerve conduction was checked 5 times prior to extract application. On this particular vagus nerve preparation (fig. 9), 7 nonalkaloid in NaOH fraction trials from 4 alfalfa samples (2 bloat-producing) showed inhibition of nerve conduction.

A solution of KCl (200 mEq./liter) blocked vagus conduction in 50 seconds and the duration of block was 11.0 minutes. No attempt was made to determine the lowest effective concentration of KCl solution.

Mouse Injections.—Of the 171 mice used for this part of the study, 36 died as a result of injections. Sixteen mice were injected with aqueous residues and all died; 13 died from injections of first 24-hour diffusates; 6 died from nondiffusible fractions; and 1 died from a first 24-hour diffusate through cation exchange resin.

Five of 10 mice injected with nonalkaloid in NaOH fractions showed some depression following injection. After approximately 1 hour they recovered and none died. Many mice (regardless of fraction tested) were depressed following injection. This was not of long duration and was thought due to pain associated with injection. Some mice responded to external stimuli (e.g., tapping with pencil) by leaping into the air or extension of the legs and tail. These mice were in the group of 36 that died.

In general, symptoms following injection were not reliable indexes of extract activity. Those mice died that still appeared depressed 3 hours after injection. Time of death following injection varied from 80 minutes to 3 days. Postmortem examination revealed no striking gross changes.

Rabbit Cornea.—Four trials with aqueous residue showed that the irritant effect of extracts prevented testing of corneal anesthesia. Therefore, rabbit cornea application was discontinued as a screening procedure and used only in later studies of nonalkaloid in NaOH extracts. During these trials, an attempt was made to see if extracts would block pupillary reflex. None of the extracts tried showed any alteration of pupillary reflex. They did, however, produce a hyperemia of the conjunctival membranes lasting about 3 hours.

Cat Blood Pressure.—Five cats were used for these studies. A large sample of frozen leaves extracted by the first modification of the original procedure provided all of the material for test in the cat. An attempt was made in 3 cats to determine whether nonalkaloid in NaOH extract could enhance the blood pressure response of epinephrine given intravenously. Epinephrine in 2- and 4-μg. doses produced an easily measurable rise in blood pressure. Thirteen attempts in 3 cats, using 2- and 4-ml. doses of extract, prolonged the time required for blood pressure to return to normal following administration of epinephrine, but this was also shown to be true for pentobarbital sodium injection. A delay between 2 consecutive epinephrine injections after a series of evenly spaced injections produced a similar prolongation of epinephrine effect.

Four trials in 2 cats, using 2 ml. of extract alone, showed that extract produced a transitory rise and fall in blood pressure followed by a rise and then a gradual return of blood pressure to preinjection values.

Bovine Injections.—Using various fractions of aqueous residues for intravenous administration to 5 cows, 45 trials were made. Fractions studied were nondiffusible, first 24-hour diffusate, and first 24-hour diffusate through cation exchange resin. Only nondiffusible fractions consistently produced symptoms.

The following symptoms were seen: dyspnea, hyperpnea, rise in rectal temperature, muscular tremors, rumen inhibition, coughing, and pilo-erection. The ECG traces showed no significant changes. A pronounced neutrophilic leukopenia was

observed in 2 cows with symptoms. White cell counts were normal at 24 hours post-injection. Symptoms lasted 2 to 5 hours. Precipitation with ammonium sulfate removed active materials from nondiffusible fractions. Extract dosage varied from 3 to 20 ml. The amount required to produce symptoms was not consistent. The above symptoms were similar to those described for anaphylactoid reactions following intravenous administration of foreign proteins.

Discussion

The individual animal susceptibility to bloat and failure to find bloat in cattle on pastures containing a high percentage of grass, noted in this study, have been reported previously.[3, 4, 19, 22] Finding of bloat in cattle on 7 unfertilized pastures, of a total of 8, deserves further study. No interpretation could be made from these limited data.

The lack of difference between concentrations of inhibitory material in chloroform extracts from bloat-producing and nonbloat-producing pastures should be considered with some reserve. It may well be that the sampling technique used was not the same as that of a grazing cow, that samples were collected from plants that were nonbloat-producing even though harvested in bloat-producing pastures, that components of the plants were altered during storage or that the extraction procedure selected materials from the plants in an entirely different manner than would a cow.

It appeared that the nonalkaloid in NaOH extract had properties similar to flavonoids. Since Ferguson *et al.*[11] had already described a smooth muscle-inhibiting compound in alfalfa and found it to be tricin,[11] comparisons of ultraviolet spectrums and solubility properties were made. These data would indicate that the biologically active material in the extract was not tricin.

The use of pressed juices and aqueous extracts of alfalfa in small animal bioassays was not satisfactory. Considering the high potassium concentrations in legumes and the sensitivity, in general, of bioassay techniques to electrolyte imbalances, it would appear difficult to interpret responses of bioassays to solutions of unknown electrolyte content.

The ability of nonalkaloid in NaOH extract to enhance epinephrine action on rabbit uterus has been noted. Several flavonoids have been shown to have this property also.[7, 21] The ability of this extract to enhance the response of rabbit uterus to histamine has not been found reported elsewhere with study of flavonoids.

Inhibition of turtle nerve conduction by nonalkaloid in NaOH extract raises the question as to whether the same phenomenon could occur in intact animals.

Mice, too, were susceptible to extracts containing high salt concentrations. In the present study, symptoms exhibited by mice following injection were difficult to interpret. At least 5 mice should have been injected with each extract. This practice was precluded by insufficient volume of extract obtained with each extraction series.

The irritant effects of some extracts prevented evaluation of possible corneal anesthesia. Study of pupillary reflexes was possible, but nonalkaloid in NaOH extract did not show parasympatholytic activity when tested in this manner.

The cat blood pressure response was not unlike that seen for pituitrin.[2] These results show that the extract could produce physiologic changes *in vivo*.

Appearance of anaphylactoid symptoms in cattle with small doses of nondiffusible extract when injected intravenously would indicate that the extract was quite potent. However, the intravenous injections of vaccines, foreign proteins or albumoses produce similar symptoms.[31] It would not be unreasonable to believe that symptoms seen in the present study and observed by others [8, 29] were due to compounds classified as albumoses.[13]

Despite finding an active extract from alfalfa leaves which inhibited isolated rabbit ileum motility and turtle vagus nerve conduction and which produced other easily demonstrable biologic actions, the critical question as to what influence this extract might have on cattle has not been answered. The question of whether or not the material occurs in higher concentration in bloat-producing plants than in nonbloat-producing plants remains unanswered. The use of small animal bioassays has proved to be of limited value in determining whether alfalfa contains bloat-producing chemicals which exert their effects primarily by inhibiting ruminoreticular motility and eructation.

SUMMARY

An attempt was made to develop methods for assay of materials in alfalfa plants which may conceivably be responsible in part for the development of the symptoms of acute legume bloat in cattle. A systematic extraction procedure applied to alfalfa leaf samples harvested from bloat-producing and nonbloat-producing pastures was followed by chemical and biologic assays of the resulting fractions.

Bloating in cattle occurred under a variety of conditions but only when alfalfa percentage was high. Daily strip rotation management of pastures provided reasonably well-defined samples. Other grazing practices were not suitable for sampling pastures.

The results of biologic assays indicated that aqueous residues, alfalfa juices, and rumen liquids were not suitable in crude forms for determining factors causing bloat. The data suggested that electrolytes markedly interfered with interpretations. The presence of materials resembling albumoses in aqueous residues appeared to be responsible for anaphylactoid symptoms resulting from intravenous injection of nondiffusible fractions in adult cattle.

Steam distillates and alkaloid fractions showed no biologic activity. A nonalkaloid, chloroform-soluble material which exhibited a number of physiologic effects was found in all samples tested. The chemical and biologic properties of the nonalkaloid extract showed that the material resembled flavonoids but was not tricin.

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SUMMARIO IN INTERLINGUA

Essayage Biologic de Extractos de Specimens de Lucerna ab Pasturas Productori de Tympania

Esseva interprendite le tentativa de disveloppar methodos pro le essayage de materiales in plantas de lucerna que es possibilemente responsabile al minus in parte pro le causation de symptomas de acute tympania leguminose in bestial. Un systematic procedimento de extraction, applicate a specimens de folios de lucerna ab pasturas productori de tympania e ab pasturas non-productori de tympania, esseva sequite per essayage chimic e biologic del resultante fractiones.

Tympania in bestial occurreva sub varie conditiones sed solmente quando le procentage de lucerna esseva alte. Un programma de rotation de bandas de pastura ab die in die provideva satis ben definite specimens. Altere methodos de pasturage non esseva satisfacente in le obtention de specimens.

Le resultatos del essayage biologic indicava que residuos aquose, succos de lucerna, e liquidos del rumine non esseva usabile in forma crude pro determinar le factores que causa tympania. Le datos suggere que le electrolytos interfere marcatemente con le interpretation. Le presentia de materiales resimilante albumosas in le residuos aquose pareva esser responsabile pro le symptomas anaphylactoidic que resultava ab le injection intravenose de non-diffundibile fractiones in bestial adulte.

Distillatos a vapor e fractiones alcaloidic monstrava nulle activitate biologic. Un material non-alcaloidic e solubile in chloroformo, le qual exhibiva un numero de effectos physiologic esseva trovate in omne le specimens testate. Le proprietates chimic e biologic del extracto non-alcaloidic monstrava que le material esseva simile al flavonoides sed que illo non esseva tricina.

Studies on the Morphology and Respiration of *Anaplasma marginale*

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BOYNTON [2] was among the first to notice that some of the marginal bodies found in erythrocytes in bovine anaplasmosis may appear to have a comet-like tail. He suggested that the tail might be a part of the erythrocyte substance drawn to the bodies. Franklin and Redmond [5] described projections or tails extending from certain anaplasma bodies in stained blood films from 5 infected animals. They could be found only within a very limited period (1 to 4 days) during the acute phase of the disease.

Phase-contrast microscopy of the erythrocytes of animals infected with anaplasmosis might provide interesting information concerning the morphology of the intracellular bodies; at the time this work was started, such studies had not been reported. Quite recently, Espana *et al.*[4] described the results of phase contrast and electron microscopic observations of erythrocyte stroma from the blood of animals infected with anaplasmosis. By both methods, they found that the hemolyzed cells contained structures described as ring, match, comet, and dumbbell forms.

An understanding of the metabolism of malarial plasmodia has been achieved through a study of respiration and biochemical activities of parasitized erythrocytes from the blood of infected monkeys.[8] Hence, it appeared that a similar approach might be worthwhile in the case of Anaplasma. With this purpose in mind, the respiratory rates of blood specimens from calves in the acute stage of anaplasmosis were measured and compared with similar rates for normal bovine blood.

MATERIALS AND METHODS

Experimental Animals.—Eleven calves, 4 to 18 months old, were splenectomized at least 1 month prior to inoculation, except calf 405, which was splenectomized after inoculation. All animals were held in screened quarters before being inoculated and afterwards were moved to insect-proof stalls. This work was done in an area where anaplasmosis is not enzootic.

Experimental Infection with Anaplasma marginale.—The isolate of *Anaplasma marginale* employed was originally obtained from animal 80 in the Oregon State College beef herd and has been maintained in carrier animals since that time. The carrier used to provide infectious blood was kept in an insect-proof stall. The isolate of the infectious agent had only moderate virulence; only 2 of 11 animals infected during the course of this work succumbed to the infection.

Experimental animals were infected in some cases by the subcutaneous injection of 5.0 ml. of infectious blood. In other cases, to reduce the incubation period to a minimum, infection was induced by the transfusion of 350 to 400 ml. of a mixture of equal parts of infectious blood and Alsever's solution.

A second infectious agent was observed in the carrier blood. This agent was sometimes found in the blood of experimentally infected animals, appearing as small coccoid or granular bodies attached to the surface of erythrocytes or free in the plasma. Such bodies, when they were observed, appeared earlier than Anaplasma, and could be distinguished from the latter in stained blood films and in unstained wet films under the phase microscope. Blood specimens observed to contain these forms were excluded from the results reported here.

Counting Anaplasma in Stained Films.—Blood smears were prepared from oxalated, freshly drawn blood. These were stained by the Giemsa method,[10] and the percentage of erythrocytes containing Anaplasma was determined from a count of 500 cells.

Examination of Blood Specimens by Phase Microscopy.—Specimens of oxalated or, in some cases, defibrinated blood were used for examination by phase-contrast microscopy. Hemolysis of the cells, required for the visualization of intracellular structures, was accomplished by quick freezing of the blood by partial immersion of the tube in ethanol at about −60 C. Specimens could then be stored at that temperature until needed. They were thawed quickly in water at about 40 C. Storage of defibrinated blood at 4 to 6 C. for a week or more was also found to result in hemolysis of a sufficient number of infected cells to give a satisfactory preparation. In either case, wet films were prepared by placing a small drop of the hemolyzed

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specimen on a slide and allowing it to spread out under a No. 1 cover slip. If dilution of the blood was required, a small drop of Hanks' balanced salt solution was mixed with the blood on the slide. If photomicrographs were to be made, the blood was mixed with a drop of 10 per cent gelatin solution at about 45 C. on the slide to overcome Brownian movement. With all preparations, the cover slip was firmly pressed down and the edges sealed with paraffin.

A Leitz Ortholux microscope, equipped with a Heine phase-contrast condenser adjusted to give dark-phase contrast, and a 90X oil-immersion phase-contrast objective was used in examining blood specimens. Photomicrographs were taken with a Leica camera attachment, using Adox KB14 35-mm. film.

The percentage of infected erythrocytes in wet films was determined by counting 500 stromas and recording the number containing tailed or dumbbell forms.

Counting Reticulocytes.—Blood smears were stained with brilliant cresyl blue,[6] and the number of reticulocytes occurring in a count of 500 cells was determined.

Measurement of Respiration.—Blood specimens to be used for measurement of respiration were drawn aseptically and defibrinated. The blood was centrifuged at about 2,000 r.p.m. for 20 to 30 minutes to concentrate the erythrocytes, and the supernatant serum was removed. The concentrated cells were diluted for measurement of oxygen consumption by mixing, in a sterile flask, 1 volume of the cells with 2 volumes of a phosphate-buffered saline solution (pH 7.6) shown to be isotonic for bovine red blood cells. The mixture of cells and buffered saline solution was tested for sterility by inoculating 1-ml. volumes into tubes of thioglycollate broth and incubating for 7 days at 37 C.

Three milliliters of the diluted blood was then added to each of 3 single sidearm Warburg flasks. Triplicate flasks were run for each set of conditions in most experiments. To the center well of each flask was added 0.2 ml. of Pardee's buffer[14, 18] adjusted to maintain an atmosphere containing about 3 per cent CO_2 in the flasks after equilibrium was reached. Three control flasks contained 3.0 ml. of buffered saline solution in the main reservoir and 0.2 ml. of Pardee's buffer in the center well to provide a correction for any slight pressure changes due to the buffer itself. The thermobarometer flask contained 3.0 ml. of distilled water only. A small roll of filter paper was placed in the center well of each flask in the usual way, and the flask was attached to its manometer. Each assembly was then aerated with alveolar air by blowing through the side arm stopper for at least 30 seconds to hasten the establishment of a CO_2-containing atmosphere. The flasks were then placed on a shaking apparatus in the water bath, which was maintained at 38 C. An equilibriation period of 45 minutes was employed in the first experiments, but this was later increased to 90 minutes to permit establishment of a more stable equilibrium between the buffer solution and the gas phase. Readings of all manometers were then taken at 15-minute intervals over a 2-hour period.

At the end of each run, flasks were stored in the refrigerator until the following day. Packed cell volume measurements were then made on the diluted blood from 2 flasks in each experimental group. Wintrobe hematocrit tubes were used, with centrifugation at about 700 g for 1 hour.

Calculation of Oxygen Consumption.—Oxygen consumption for each blood specimen was calculated in terms of cubic millimeters of O_2 per hour per 1.0 ml. of packed red blood cells. The oxygen consumption per flask per hour was obtained by multiplying the observed drop in pressure by the flask constant. The mean value was then found for the triplicate flasks. If small pressure changes were noticed in the Pardee buffer control flasks, the mean change was applied as a correction to the mean value for oxygen consumption in the 3 flasks containing the blood specimen. The final calculation was then made according to the following formula:

$$\text{cmm. of } O_2/\text{hr./ml. p.c.v.} = \frac{\text{mean } O_2 \text{ consumption/flask/hr. in cmm.}}{3 \times \text{ml. packed cells/ml. diluted blood/flask}}$$

The denominator in this fraction derives from the fact that each flask contained 3.0 ml. of diluted blood. The "ml. of packed cells/ml. of diluted blood" was obtained from the hematocrit reading made on the flask contents.

Pardee's Buffer.—The buffer used for maintaining a CO_2-containing atmosphere was composed of the following:[14, 18]

Diethanolamine
(60% by volume in distilled water)....10.0 ml.
6N HCl....4.1 ml.
$KHCO_3$ (powder)....3.0 Gm.
Thiourea....0.015 Gm.
Distilled water....0.9 ml.

Phosphate-Buffered Saline Solution.—The phosphate-buffered saline solution had the following composition:[11]

	Gm.
NaCl	7.5
KCl	0.75
Na_2HPO_4 (anhydrous)	0.80
KH_2PO_4	0.12
K_2HPO_4	0.50

Distilled water to make 1,000 ml.

The pH of the solution was 7.6 ± 0.05. It was sterilized by Seitz filtration. The solution was isotonic for bovine erythrocytes.

EXPERIMENTAL RESULTS

MORPHOLOGY OF ANAPLASMA MARGINALE

Blood specimens from each of 9 infected calves were drawn 2 or 3 times a week during the period when the red blood cells containing Anaplasma exceeded 1 per cent of the total. No less than 6 microscopic

examinations were made on each animal during this period. Blood from 9 normal calves was taken at different times and examined in the same manner.

In lysed cells or stromas in specimens of blood from calves in the acute stage of infection, intracellular structures were seen which were considerably different from the round marginal bodies which are found in the red blood cells in blood films stained by the Giemsa method. The form most frequently observed possessed 2 parts, 1 of which was more or less spherical and can be conveniently referred to as the head portion, located near the periphery of the cell and measuring about 0.5 to 1.5 μ in diameter. Attached to the head and extending completely across the cell was a tail-like appendage. This appeared to be a thin, wedge-shaped structure about 0.3 or 0.4 μ thick near its attachment to the head, tapering to a point at its opposite end. The length of the tail varied from 1.3 to 3.5 μ. Stromas containing this intracellular form are shown (fig. 1, 4). In other stromas, the tail appeared as a broad, thin structure about 1.0 to 1.8 μ wide and of low density, becoming thinnest near the free end and suggesting a comet (fig. 3, 5). It was first thought that the pointed tail and the comet forms were different, but it was found that the difference was only apparent and was due to the position in which the entire body was observed inside the stroma. The tail was a broad membranous structure and, if the narrow edge was viewed, it appeared thin and tapering to a point. However, if the body was rotated through about 90 degrees, the broad, flat surface of the tail was seen; decreasing thickness contributed to the comet-like appearance. This relationship between the 2 apparently different forms was proven repeatedly by observing 1 form change into the other as the entire body rotated inside the stroma.

In the majority of these intracellular structures, the head appeared as a single body, but in some cases it could be seen to be divided into a number of spherical or somewhat elongated segments. Heads containing 2 to 4 segments were common (fig. 8). As the number of segments increased, accurate counting became more difficult, but it appeared that the number could be as high as 6 or 7 (fig. 10), though such forms were uncommon.

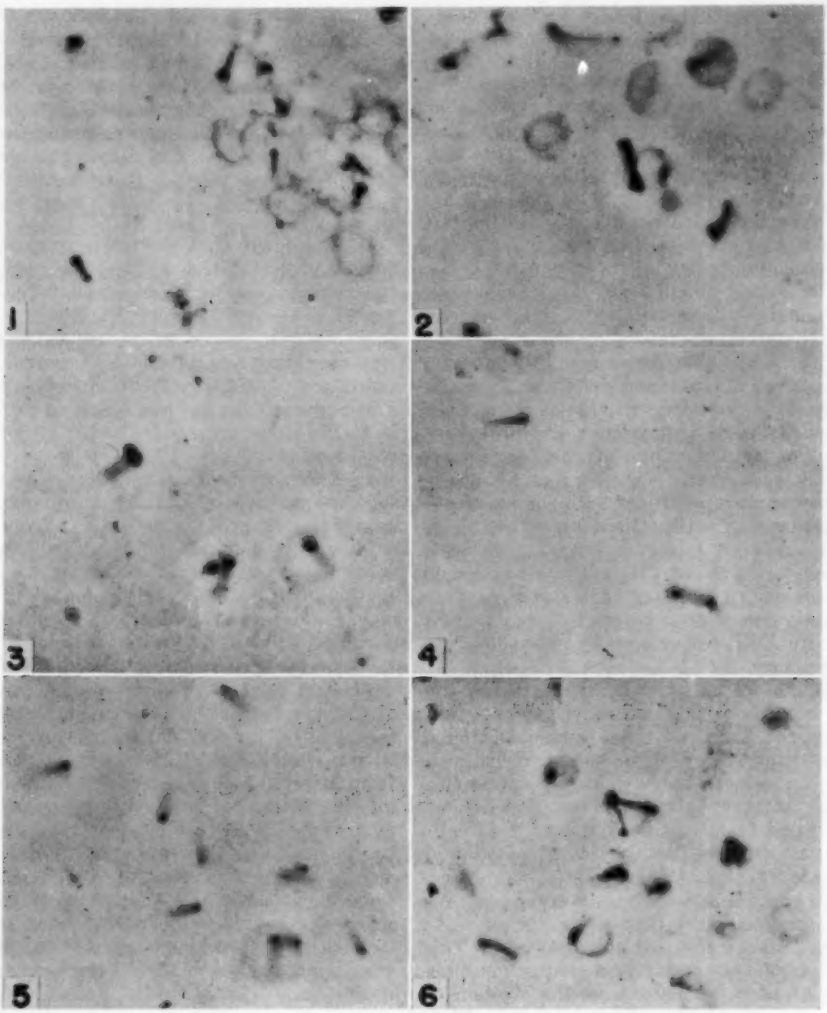
Occasionally in infected stroma, the single or multiple head parts appeared to have become separated from the tail portion, and in others spherical bodies were seen which closely resembled the heads, but were apparently without tails.

Another morphologic variant found in some of the infected cells can best be described as a dumbbell form (fig. 2, 4, 7) because it consisted of 2 heads located near the periphery of a red blood cell stroma, but at opposite sides, which were connected by a thin band of material having the same appearance as that forming the tail of the comet forms. In these forms also, the heads were observed to be either single or divided into a number of segments (fig. 7). The diameter of the heads in these forms had the same range of variation as in the comet forms, and the over-all length of the dumbbell forms varied from 3.5 to 5.0 μ. They were much less numerous than the tailed forms. In an instance where a count was made on blood from an animal at the height of the infection, dumbbell forms constituted 11 per cent of all forms observed.

No clear-cut association of any of the morphologic variations with a particular stage of the infection in the animal was observed. The tailed forms, usually the first to be observed by phase microscopy, were seen at the stage where the marginal bodies in Giemsa-stained films were first becoming evident. At the peak of the erythrocyte infection, most of the variants could be found.

In erythrocyte stroma of normal bovine blood, no structures were seen which resembled any of those described for erythrocytes from infected animals.

Continued study of the tailed and dumbbell forms in infected blood suggested that the head portions of these forms were probably identical with the marginal bodies seen in Giemsa-stained blood films. This was indicated both by the marginal location of the heads in the cell stromas and by the similar range of size variation noticed in these bodies and in the stained bodies. Furthermore, the marginal position of the stained bodies becomes readily understandable on this basis, as the length of the tail and its position inside the cell would automatically locate the head at some point near the periphery of the cell. To obtain further information on this point,



Various forms of Anaplasma bodies seen by phase-contrast microscopy in the stroma of hemolyzed erythrocytes from the blood of calves during the acute stage of anaplasmosis. x 1125.

Fig. 1—Bodies in stroma showing round or oval heads and wedge-shaped pointed tails, seen when thin edges of tails are in view.

Fig. 2 and 4—Dumbbell forms with 2 heads joined by interconnecting band.

Fig. 3 and 5—Comet-like forms showing relatively dense heads and wide tails of decreasing thickness, seen when tails are viewed from broad flat side.

Fig. 6—Uncommon form in which 2 dumbbells in single stroma appear to be fused at 1 end.

comparisons were made of the percentage of red blood cells containing marginal bodies stained by Giemsa and the percentage of lysed stroma containing tailed and dumbbell forms visible by phase-contrast microscopy, using the same specimen of blood. Such comparisons were made at intervals of 2 or 3 days during the acute stage of infection in 7 infected calves. In general, there was fairly good agreement

in the percentages of infected erythrocytes determined by the 2 methods. In some individual counts, considerable discrepancy was noticed, but this is not surprising in view of the large sampling errors associated with differential counting of this type. The comparative data obtained during the rise and fall of percentages of these bodies in the red blood cells of 2 of the animals studied are shown (fig. 13). The incubation period was shortened in calf 411 by the transfusion of 350 ml. of 1:2 dilution of carrier blood, while calf 415 was given the usual 5-ml. inoculation. It is evident that the results obtained by the 2 methods are in reasonable agreement throughout the course of infection in these animals. These data, together with those previously recorded, were interpreted as indicating that the head portions of the forms seen in the lysed erythrocyte stroma by phase microscopy are identical with the marginal bodies seen in the Giemsa-stained blood films. As the latter are commonly presumed to represent the infectious agent of the disease, it appears that the same presumption may now be applied to the tail and dumbbell forms seen by phase-contrast microscopy.

Espana *et al.*[4] found structures in the red blood cell stroma which were essentially the same as those described here; in addition, they described a ring form which was not observed in our specimens. These workers also reported motility of the tailed forms inside the hemolyzed cells of specimens of freshly drawn blood and considered that this indicated the infectious agent is a truly motile organism. We have noticed also that these forms move about inside the infected stroma. This movement may result in a change of direction of these structures in the stroma, or in rotation, and may cause the head or tail to move in or out of focus. Structures seem to be more active in freshly drawn blood specimens. However, the Brownian movement of particles inside the hemolyzed cells, and of the cells themselves, is quite active in these preparations, and we have not been able to convince ourselves that the movement shown by the tailed forms is due to self-propulsion, and is not molecular in origin.

Attempts were made to stain the tailed forms in the hemolyzed erythrocytes, but these were, in general, unsuccessful. Among the methods tried were Giemsa, Wright's stain, Leifson modified flagella stain, celestine blue with eosin, the Feulgen stain, and auramine O. It was noticed that dilution of infected blood with water for hemolysis apparently rendered the tails no longer visible by phase-contrast microscopy.

RESPIRATION OF ANAPLASMA-INFECTED BLOOD

It is known that normal erythrocytes have slight oxidative activity and employ anaerobic glycolytic processes as their chief source of energy. However, erythrocytes parasitized by malarial parasites have an active oxidative metabolism. Oxygen consumption of monkey cells infected with *Plasmodium knowlesi* was found to be from 25 to 100 times greater than that of normal red blood cells.[8]

In view of the uncertainty concerning the true nature of the agent of anaplasmosis, and the knowledge gained from the studies with malaria, it appeared worthwhile to determine, if possible, whether erythrocytes infected with *A. marginale* may also consume oxygen at a rate comparable to that found with malarial blood.

The same calves infected with anaplasmosis and used in the morphologic studies of the agent also served as a source of infected blood for respiration measurements. Blood specimens were drawn aseptically from infected animals at various times during the acute phase of the infection and defibrinated. At the same time, similar specimens were drawn from normal animals to serve as a basis of comparison in each experiment. In many cases, calves selected for splenectomy and later infection with anaplasmosis were used as sources of normal blood before they had been inoculated.

Two or more measurements of oxygen consumption were made on the blood of each of 6 infected calves during the acute stage of the disease. In all, 18 comparisons were made of the respiratory rate of infected and normal bovine blood.

At the time most of this work was in progress, it was not appreciated that reticulocytes respire at a rate many times greater than that of mature erythrocytes.[20] Considering that a severe anemia develops during the course of anaplasmosis, it appears probable that, at some stage in the disease, reticulocytes will appear in the circulating blood and might cause a sig-

nificant increase in the respiratory rate of the blood. Machado[9] studied the occurrence of reticulocytes in the blood of cattle experimentally infected with anaplasmosis and piroplasmosis. No reticulocytes were observed during the first week after Anaplasma appeared in the erythrocytes; they were first seen when the proportions of Anaplasma-infected cells had reached maximal values, and hematocrit values had dropped significantly.

In order to obtain some further information on this point, calf 415 was inoculated with 5 cc. of blood from an anaplasmosis carrier animal. Blood specimens were taken at frequent intervals from the seventeenth until the 55th day after inoculation. Anaplasma-infected cells first reached a level of 1.0 per cent of the total on the 28th day after inoculation and increased to a maximum of 25.0 per cent on the 38th day. No reticulocytes were found in any blood specimens until the 38th day, when a count indicated an incidence of about 0.2 per cent of the circulating red blood cells. This value rose to a maximum of 1.9 per cent on the 45th day and dropped to 0 again by the 55th day. On the 38th day, when reticulocytes were first seen, the hematocrit value had dropped to 14.8 per cent from an initial value of 21.0 per cent. It thus appeared that the observations regarding reticulocytosis in this 1 infected animal were in reasonable agreement with those reported by Machado.[9] In both cases, the first appearance of reticulocytes coincided with the maximum incidence of infected red blood cells, and did not occur until a marked degree of anemia had developed. Thus, the limited data available suggested that the blood of animals infected with anaplasmosis probably does not contain reticulocytes during the first week after the Anaplasma appear in significant numbers in the red blood cells (1 per cent or more of cells infected).

On the basis of this information, respiration measurements on all blood specimens drawn more than 7 days after the infected erythrocytes first reached the 1 per cent level were excluded from consideration. It seemed probable that in this way any significant influence of reticulocytes on the respiratory rate could be eliminated.

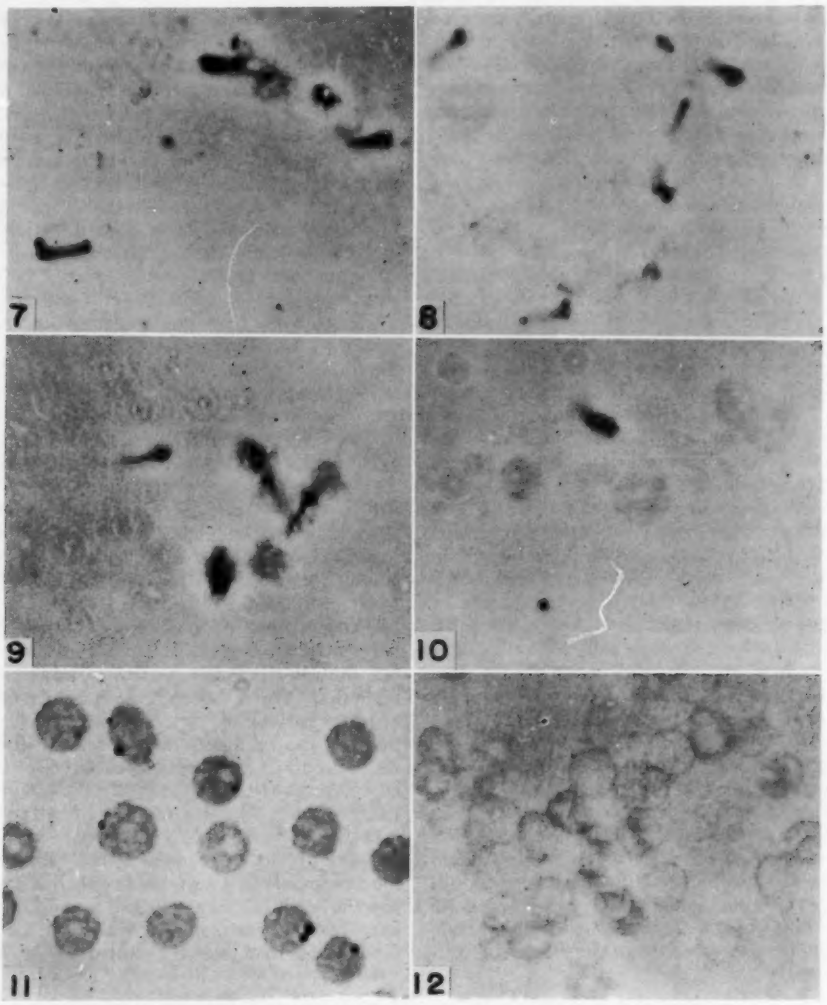
The results of oxygen consumption measurements on specimens taken during the first 7-day period of the red blood cell infection and on normal control specimens are shown (table 1). Despite variations in the results of individual experiments, 2 conclusions seem to be justified by the data. The mean respiratory rate for all of the infected blood specimens is slightly more than double the rate for normal bovine blood. Inspection of the data suggests that this difference is real, and when the results were compared by means of the "t" test for comparison of means,[10] the difference was found to be highly significant. On the assumption that any possible influence of reticulocytes has been ruled out, it then appears probable that the increased respiratory rate in infected blood is due to its content of infected erythrocytes. Considering all the infected blood specimens listed (table 1), the mean percentage of infected cells was 10.3 per cent, and the mean respiratory rate was 9.1 cmm. per hour per milliliter of packed cell volume. On the basis of 14 erythrocyte counts and hematocrit measurements made on diluted blood used to measure respiration in War-

TABLE 1—Respiration of Anaplasma-Infected and Normal Bovine Blood

Exp. No.	Infected animal No.	% of red cells infected	Normal animal No.	cmm. O_2/hr./ml. p.c.v.*	
				Infected blood	Normal blood
6	407	11.9	409	16.6	8.0
9	409	4.2	411	9.1	4.2
10	409	9.0	411	7.1	4.6
15	411	37.8	415	6.5	4.5
18	410	4.3	H-11	8.6	0.5
19	410	8.3	358	9.1	3.6
21	412	5.3	H-70	4.6	3.6
22	412	6.3	415	8.0	4.8
21	413	5.2	H-70	9.7	3.6**
22	413	10.9	415	11.4	4.8**
Mean				9.1	4.2

* Expressed as cubic milliliters of O_2 consumed per milliliter of packed erythrocytes per hour. Values were determined over a 2-hour period.

** These normal values are the same as the 2 immediately above, but represent the proper controls for the infected blood specimens indicated. They were used only once in calculating the mean for normal blood.



Additional forms of Anaplasma bodies seen by phase-contrast microscopy. x 1125.

Fig. 7—Dumbbell form in erythrocyte stroma in which 1 of heads appears to have 2 parts.

Fig. 8—Comet-like forms in which heads seem to have 2 to 4 parts.

Fig. 9—Anaplasma body freed from erythrocyte stroma by treatment of blood specimen with 3.7 per cent saponin.

Fig. 10—Comet-like form with multiple head consisting of 6 or 7 parts.

Fig. 11—Typical marginal Anaplasma bodies in erythrocytes in blood film stained by Giemsa's method.

Fig. 12—Erythrocyte stroma in specimen of normal calf blood hemolyzed by freezing and thawing.

burg flasks, 1.0 ml. of packed cells was equivalent to 23.0 x 10^9 cells. If, in the infected blood, an average of 89.7 per cent of the cells were respiring at the rate of normal blood, or 4.2 cmm./hr./ml. p.c.v., the infected cells (10.3%) must have respired at the rate of 51.8 cmm./hr./ml. p.c.v. to produce the observed rate of 9.1

cmm. For the infected cells, then, this rate is equivalent to 51.8 cmm. per hour per 23.0 x 10^9 cells. If it is assumed that both cells and Anaplasma are utilizing oxygen, then the oxygen consumption of the erythrocytes alone, or 4.2 cmm. per hour, should be deducted from this value to give the respiration of the Anaplasma alone. This gives 47.6 cmm. per hour per 23.0 x 10^9 infected cells, a rate slightly more than 10 times that of the normal cells. If it is assumed that the average infected cell has 1 Anaplasma body, then the respiratory rate becomes 2.07 cmm. per hour per 10^9 Anaplasma. Although this is a little more than 10 times the rate for the erythrocytes alone, it is far below the values reported for oxygen consumption by malarial parasites and other parasitic protozoa.[19] Thus, the O_2 consumed per hour per 10^9 parasites for *P. knowlesi* varied from 80 to 340 cmm., depending on the stage of development of the organisms. For *Trypanosoma gambiense*, the rate was 1,700 cmm./hr./10^9 parasites; for *Trichomonas foetus*, 2,150 cmm./hr.; and for *Leishmania donovani*, 440 cmm./hr. It appears, then, that any oxygen consumption attributable to the Anaplasma is small and not at all comparable to that found with recognized forms of parasitic protozoa, including the plasmodia, which were also studied as parasitized erythrocytes.

Discussion

It is interesting to consider whether these and other recent findings shed any new light on the biological nature of *A. marginale*. It now seems apparent that the abnormal structures found in the erythrocytes of infected animals, and presumed to represent the etiologic agent, have a more complex morphology than previously thought. However, the relationship between the various forms observed is, in most cases, unclear and will require much more work for clarification. Little is known about the method of reproduction. Lotze and Yiengst,[7] working with stained films of blood previously treated with dilute acetic acid, observed that certain of the marginal bodies appeared to consist of groups or clusters of as many as 8 small spherical bodies, which they regarded as the products of a multiple division process. The observations reported here may be compatible with this view. Thus, the multiple components of the heads of the comet forms (fig. 8, 10) may possibly represent the products of such a process. The relationship of the dumbbell forms to the tailed forms with single heads is far from clear. The dumbbells could represent a stage in a developmental cycle, or conceivably might result from the fusion of 2 comet forms in a sexual type of reproduction. No extraerythrocytic structures which might represent the form in which the agent invades the erythrocyte were recognized in this study.

There appear to be 3 current theories regarding the biological nature of *A. marginale*. It is variously regarded as a protozoon parasite, a virus, and, according to "Bergey's Manual of Determinative Bacteriology,"[73] a separate family of the order Rickettsiales. The morphologic findings could be compatible with the protozoon theory. However, the extremely small oxygen consumption revealed by the respiration studies seems to indicate that the Anaplasma have a different type of metabolism

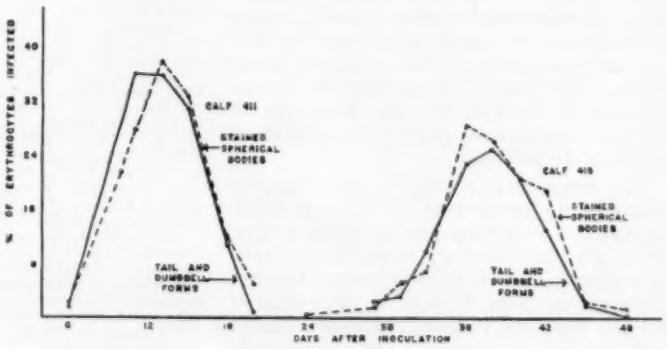


Fig. 13—Comparative counts of tail forms, seen by phase-contrast microscopy, and of stained marginal bodies in erythrocytes of calves affected with anaplasmosis.

from that of various recognized parasitic protozoa, all of which have marked oxidative activity.[19]

If the intracellular forms described represent the infectious agent, their characteristics do not seem to support the virus theory. There are no agents presently classified among the viruses which are in the size range of the tailed or dumbbell forms. In general, infection of tissues by a number of different animal viruses has usually been observed to cause no increase in oxygen consumption by such tissues.[1, 12, 13, 15, 17] Thus, neither the morphologic nor the respiration data support the idea that this agent is a virus.

In the light of our very limited knowledge of *A. marginale*, it would appear most reasonable to regard it as a parasitic organism sufficiently different from other forms to justify separate family status under the order Rickettsiales. In this way, the morphologic characteristics become those characterizing the family, and the information reported on respiration does not have to be regarded as an exception to the usual findings of other members of a group.

It seems apparent that the examination of blood films by phase-contrast microscopy may be an alternative method for the diagnosis of bovine anaplasmosis during the periods when the erythrocytes are infected. Because the morphologic forms observed by this method are so distinctive, recognition of the disease may be somewhat facilitated in comparison to the conventional stained-film technique.

SUMMARY

Using the phase-contrast microscope, hemolyzed erythrocytes of calves in the acute stage of anaplasmosis were found to have intracellular structures more complex than the marginal bodies seen in stained blood films. This procedure appears to be an alternative method for the diagnosis of bovine anaplasmosis during the period of erythrocyte infection.

The structures, consisting of single or multiple round or oval head parts which appeared to represent the marginal bodies, were attached to flat, membranous tails that decreased in thickness toward the end distal to the head. When the thin edge of the tail was seen, it appeared wedge-shaped and tapered to a point. When the wide flat side was seen, it appeared comet-like. Dumbbell forms with a head at each end of an interconnecting band were also seen.

A study was made of the respiratory rate of blood from anaplosmosis-infected calves during the first week of the period in which the percentage of infected erythrocytes was rising rapidly. On the basis of 10 experiments involving 6 infected animals and a similar number of normal controls, the mean rate of oxygen consumption for the infected blood was a little more than double that of the normal blood. This was a significant difference, but if it is assumed that the increase was due to the Anaplasma bodies present, it appears that the respiratory rate of these bodies is only a small fraction of that found for malarial plasmodia and for other parasitic protozoa.

The information reported lends support to the classification of Anaplasma as a separate family, Anaplasmataceae, of the order Rickettsiales.

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SUMMARIO IN INTERLINGUA

Studios in Re le Morphologia e le Respiration de *Anaplasma marginale*

Per medio de microscopia a contrasto de phase, il esseva constatate que hemolysate erythrocytos de vitellos in le stadio acute de anaplasmosis possede un plus complexe structura intracellular que le corpores marginal vidite in tincturate frottis de sanguine. Le technica usate pare esser un methodo alternative pro le diagnose de anaplasmosis bovin durante le periodo de infection erythrocytic.

Le structuras, consistente de solitari o multiple partes capital de conformation ronde o oval e representante (il pareva) le corpores marginal, esseva attachate a platte caudas membranose que decresceva in spissitate in le direction opposite al capite. Quando le margine tenue del cauda esseva visibile, illo pareva cuneiforme e se terminava in un puncta. Quando le large latere platte esseva visibile, illo habeva un apparentia cometari. Formas dual, con capites a ambe extremitates e un banda de interconnexion, esseva etiam incontrate.

Esseva studiate le respiration de sanguine ab vitellos inficite de anaplasmosis in le prime septimana del periodo durante le qual le procentage del afficite erythrocytos cresceva rapidemente. Super le base de 10 experimentos con 6 inficite animales e le mesme numero de normal animales de controlo, le valor medie del consumo de oxygeno per sanguine inficite esseva un pauco plus que duo vices le consumo de oxygeno per sanguine normal. Iste differentia es significative, sed si nos suppone que le augmento esseva causate per le corpores de Anaplasma presente, il pare que le intensitate respiratori de iste corpores es solmente un micre fraction de illo trovate pro plasmodios malarial e pro altere protozoos parasitic.

Le information hic reportate supporta le classification de Anaplasma como un familia separate, le familia del Anaplasmataceas, in le ordine del Rickettsiales.

The Use of Agar Plates as an Aid for the Isolation of Leptospires

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SINCE 1916, various semisolid mediums have been developed and used for the isolation of leptospires from blood, urine, kidneys, and other body tissues and fluids. Examples of such mediums are those of Vervoort, Chang, Korthof, Fletcher, and Noguchi.[2] Some type of body fluid, such as serum, was added for enrichment.

Recently, there have been a number of reports on the use of solid medium for the colonization of leptospires. Cox and Larson [4] described the growth of various strains of leptospires on an agar medium composed of tryptose-phosphate, rabbit serum, and 1 per cent agar. Larson *et al.*[7] applied this technique to enumerating leptospires in culture and, in one instance, in the blood of a guinea pig experimentally infected with *Leptospira pomona*. Kirschner and Graham [6] suggested the use of solid medium in tubes for the maintenance of stock cultures. They also suggested the use of solid medium in plates for purification of stock cultures. Goldberg and Armstrong [5] demonstrated oxidase activity of leptospires grown on solid medium. Armstrong and Goldberg,[3] in a study of the colonial morphology of 15 strains, reported six colonial types. They also concluded that the colonies were subsurface.

Epizootiologic studies in progress afforded an opportunity to use the agar plate method for primary isolation of leptospires. The objective was to determine if additional leptospiral isolates would be obtained if solid mediums were employed along with semisolid mediums, thereby serving as an ancillary method. Even though the results with the semisolid mediums are presented on a comparative basis, conclusions cannot be drawn as to the comparative sensitivity of the two methods because of the difference in the amount of inoculum used. The data are presented in this manner to show that additional isolations of leptospires were obtained by the agar plate method.

The purpose of this report is to describe the use of an agar plate method for the isolation of a number of strains of leptospires from renal tissues of rats, mice, opossums, skunks, raccoons, and calves. The identity of the strains will be stated where known. Additional data will be presented concerning isolations accomplished by the agar plate method where duplicate attempts in semisolid mediums were negative, presumably due to contamination.

MATERIALS AND METHODS

The rats, mice, raccoons, opossums, and skunks were trapped or caught alive within 100 miles of Baton Rouge, Louisiana. They were killed with carbon monoxide and exsanguinated, during which time a blood specimen was obtained. The abdominal area was shaved, cleansed thoroughly, and disinfectant (benzalkonium chloride*) was applied. A necropsy was performed with regard to asepsis, and the kidneys were removed to a sterile Petri dish with sterile instruments.

The Holstein-Friesian bull calves were 6 to 8 months old, and had been castrated. They originated from a herd that was serologically negative for leptospirosis due to *L. pomona*. These calves were experimentally infected with the strains of *L. pomona* derived from skunks. With regard for asepsis, each kidney was collected as an individual specimen and examined bacteriologically for leptospires, using semisolid mediums and the agar plate medium.

The kidneys were taken to a culture laboratory where ultraviolet lights are kept on at all times, except when the laboratory is being used. Small portions of both kidneys from each wild animal were removed with sterile instruments and pooled by grinding in mortar with pestle. No abrasives were used. Each bovine kidney was examined as a separate specimen. Sufficient Stuart's liquid medium was added to give approximately a 10 per cent suspension by volume, which was used as inoculum for the semisolid mediums. Serial tenfold dilutions of 1:100, 1:1,000, and 1:10,000 in Stuart's medium were used to inoculate the agar plates.

Medium for the agar plates was prepared as described by Cox and Larson.[4] Approximately

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* Roccal, produced by Winthrop Laboratories, Inc., New York, N.Y.

25.0 ml. was poured in sterile 15- by 100-mm. Petri dishes. Medium was not generally held longer than five days before using. One plate was inoculated with 0.1 ml. of each of the 1:100, 1:1,000, and 1:10,000 dilutions of 10 per cent kidney suspension. The inoculum was placed in the center of the Petri dish with a pipette, and distributed over the surface of the agar with a wire spreader. The plates were sealed with 1-inch adhesive tape and incubated at 30 C. in an atmosphere of high relative humidity. During the initial phase of this study, the plates were examined for leptospiral growth every other day; later, however, the examinations were reduced to once a week. Gross examinations for leptospiral growth were performed by directing the light from a microscope light through the agar plate from the back. Plates were incubated for six weeks or longer before being discarded as negative. Furthermore, dark-ground microscopy was used to examine several pieces of agar from each plate before discarding it as negative. Likewise, gross observations of positive cultures were confirmed by dark-ground microscopy. Small pieces of agar selected for examination were placed on a microscope slide and flattened under a cover slip by applying pressure with the handle of a bacteriologic loop. The microscope procedures were the same as those to be described for semisolid mediums.

The colonies of leptospires were counted by scoring the plate into quarters and making exact counts when possible. The counts were made when it appeared that no more new colonies were developing. When exact counts were impossible, colonization was recorded as too numerous to count (TNTC) or as confluent growth, whichever appeared most appropriate.

Five types of semisolid mediums were employed during the course of this study. Fletcher's basal medium containing rabbit serum and Fletcher's basal medium containing horse serum were used during the entire study. Chang's basal medium containing rabbit serum was used most of the time; whereas, Chang's basal medium containing horse serum was used about 50 per cent of the time. Modified Stuart's medium containing rabbit serum was used about 75 per cent of the time.[8] After sterilization by Seitz filtration, the respective serum was added to give a final concentration of 10 per cent. Before use, all semisolid mediums were incubated at 37 C. for 24 to 48 hours to determine sterility.

When employed, at least two tubes were inoculated with 3 to 5 drops of kidney suspension, using a syringe equipped with a 20-gauge needle. After inoculation, the mediums were incubated at 30 C. for 75 days before being discarded as negative. Periodic dark-ground examinations, made on each individual tube to determine positive cultures, utilized a binocular microscope equipped with a 45X objective and 20X eyepieces, as well as a standard dark-field condenser with a numerical aperture of 1.25. Light was furnished by a lamp housing containing a 300-watt projection bulb. To reduce heat, the light was passed through a glass receptacle containing water. Oil was used between the condenser and microscope slide, and No. 1 cover slips were used over the specimen to be examined.

The Stuart's liquid medium used differed from the original in preparation only. The basal portion was prepared from a concentrate* in 3-liter aliquots. Rabbit serum was added to give a final concentration of 10 per cent. After the pH was adjusted to 7.5, the complete medium was filtered, using a 2-liter Seitz filter equipped with an ST-3 sterilizing asbestos filter pad. The entire 3 liters was collected in a 4-liter aspirator bottle, to which tubing apparatus had been attached. Medium used to maintain antigens for the microscopic test was dispensed into sterile, screw-capped, 25- by 150-mm. culture tubes; each tube contained about 15 to 18 ml. Medium for production of absorption antigen was dispensed in 250-ml. amounts into sterile, screw-capped, 32-oz. prescription bottles. All were incubated at 37 C. for 48 hours to determine sterility.

The strains of leptospires used as antigens in the microscopic agglutination test, in the agglutination absorption test, and for the preparation of antiserums were type strains listed in a report of the Committee on Leptospirosis of the World Health Organization.[10] The Leptospira serotypes employed were: *L. ballum*, *L. canicola*, *L. icterohaemorrhagiae*, *L. bataviae*, *L. grippotyphosa*, *L. pyrogenes*, *L. autumnalis autumnalis*, *L. pomona*, *L. sejroe*, *L. hyos hyos*, *L. hardjo*, *L. semarang*, *L. djatzi*, *L. djasiman*, *L. sentot*, *L. australis A*, *L. zanoni*, *L. medanensis*, *L. javanica*, *L. andaman*, and *L. mini mini*. Additional serotypes of the *canicola*, *hebdomadis*, and *autumnalis* serogroups were employed to study some of the isolates.

Leptospiral strains used for antigens in the microscopic agglutination tests were maintained in Stuart's liquid medium and transferred every four to five days. The cultures were used as antigens when five to seven days old. Before use, cultures were examined by dark-ground microscopy for density and smoothness. If the cultures appeared too dense, they were diluted with Stuart's medium. The isolates were similarly maintained in Stuart's medium until satisfactory growth was obtained; then they were tested against known antiserums.

Antiserums were prepared according to the method described by Alexander *et al.*[1] The type strains previously listed were used. Four successive doses of ten-day-old cultures of living leptospires were inoculated, via ear veins, into rabbits at seven-day intervals. The serum was harvested seven days after the last inoculation, mixed with an equal amount of glycerine, and sterilized by Seitz filtration. Most of the antiserums employed had homologous titers of 1:100,000 in the microscopic agglutination test.

Antigens for absorption studies were prepared from six- to eight-day-old cultures grown in 250 ml. of Stuart's medium. Cultures were killed by the addition of formalin to provide a final concen-

* Difco product No. 0988-01, Difco Laboratories, Detroit, Mich.

TABLE 1—Tabulation of Animal Hosts, Culture Results, and Serotypes of Leptospires

Host species	No. examined	No. of strains isolated	Semi-solid medium only	Plates only	Both methods	Serotypes isolated*
Mus musculus (mouse)	1	1	0	0	1	*L. ballum**
Calves	6	5	0	5	0	*L. pomona* (5)**
Mephitis mephitis (striped skunk)	105	54	18	5	31	*L. pomona* (7)** (4)† *L. canicola* (5)** (3)† *L. ballum* (5)† *icterohaemorrhagiae* serogroup (1)† *hyos* serogroup (11)† *hebdomadis* serogroup (2)† Not studied (16)
Procyon lotor (raccoon)	2	1	1	0	0	*autumnalis* serogroup (1)†
Rattus rattus (rats)	4	4	0	0	4	*L. icterohaemorrhagiae* (2)** Not studied (2)
Didelphis marsupialis (opossums)	109	28	11	3	14	*L. autumnalis* (1)** *L. ballum* (1)** *hyos* serogroup (2)† *hebdomadis* serogroup (5)† Not studied (19)
Total	227	93	30	13	50	

* Number of strain is given in parentheses. ** Identity based on cross agglutinin-absorption tests with type strains. † Based on cross-agglutination patterns against serotype-specific anti-Leptospira serums.

tration of 0.25 per cent. The organisms were removed by centrifugation at 15,000 g for 15 minutes and resuspended to 1 to 2 per cent of original volume in phosphate buffered saline containing 0.25 per cent formalin. One part of a 1:10 dilution of antiserum was added to four parts of absorbing antigen, mixed thoroughly, and incubated for four hours at 37 C. A second absorption was routinely performed. In order to avoid further dilution of the antiserum, the supernatant fluid from the initial absorption was added to packed cells. The serum-cell mixture was incubated for two to four hours at 37 C., followed by overnight incubation at 30 C. The supernatant fluid was collected the following morning and tested against the antigens indicated. If necessary, additional absorptions were performed. Absorptions were considered complete when agglutinins for the absorbing strain caused less than a one-plus reaction in a dilution of 1:100. The microscopic agglutination test, using living antigen, was used to determine results. The dilution scheme employed provided for final dilutions (after addition of antigen) of 1:100, 1:300, 1:1,000, 1:3,000 to 1:100,000, and 1:300,000.

The microscopic agglutination test employed was essentially that described by Wolff.[9] In the cross-agglutination studies of the isolates against known antiserums, serum dilutions were prepared in 13- by 100-mm. test tubes with 0.85 per cent sodium chloride buffered to pH 7.4 with Sorensen's phosphate buffer. Serial tenfold dilutions were prepared so that after 0.2 ml. of each serum dilution was mixed with 0.2 ml. of antigen, final dilutions ranging from 1:30 to 1:300,000 were obtained. The serum-antigen mixtures were incubated at 30 C. for two to three hours. A binocular microscope (dry, dark field), giving a magnification of approximately X190, was used to determine the results. This magnification was obtained with a X10 objective, X15 eyepieces, and a body magnification factor of 1.25. The light source consisted of a microscope lamp equipped with a 300-watt projection bulb. The light was operated through a variable transformer so that the most favorable light intensity could be selected. The heat was reduced by placing a receptacle of water between the light source and the microscope mirror. No oil or water was used beneath the slide, and no cover slips were used. The degree of agglutination or lysis, or both, was read as one-plus (at least 25% of the leptospires agglutinated or lysed), two-plus (approximately 50%), three-plus (50 to 75%), and four-plus (75 to 100%). In the cross-agglutination studies, the end titer was recorded as the highest dilution with a four-plus reaction; however, in the absorption tests, a one-plus reaction was used to assess the end titer.

RESULTS

Ninety-three strains of leptospires were isolated from 227 specimens, the majority being from kidneys of skunks and opossums. Of the 93 strains, 88 represented original isolations; the other five were recovered from the calves that had been experimentally infected with *L. pomona*. Fifty strains were isolated by both methods, 30 strains

TABLE 2—Observations on Eleven Strains of Leptospires Isolated by the Agar Plate Method

Isolate No.	Host animal	Identity of strain	Day growth first observed	Description	
				Plate No.†	Growth
LSU 967	*Didelphis marsupialis* (opossum)	*hebdomadis*** serogroup	12	I	TNTC‡
				II	200 colonies
				III	2 colonies
LSU 1013	*Didelphis marsupialis* (opossum)	*hyos*** serogroup	10	I & II	TNTC
				III	64 colonies
LSU 1024	*Didelphis marsupialis* (opossum)	*hebdomadis*** serogroup	25	I	one colony that became very large
				II & III	negative
LSU 1056	*Mus musculus* (mouse)	*L. ballum**	14	I	confluent growth
				II	200 colonies
				III	contaminated
LSU 1066	*Rattus rattus* (rat)	*L. icterohaemorrhagiae**	16	I & II	TNTC
				III	90 colonies
LSU 1067	*Didelphis marsupialis* (opossum)	*hebdomadis*** serogroup	16	I	TNTC
				II	120 colonies
				III	12 colonies
LSU 1073	*Mephitis mephitis* (striped skunk)	*L. pomona**	17	I	growth confluent, contaminant surrounded by clear zone
				II	growth confluent
				III	3 areas of confluent growth; no colonies
LSU 1094	*Mephitis mephitis* (striped skunk)	*hebdomadis*** serogroup	13	I	30 colonies; contaminant present
				II	TNTC
				III	33 colonies
LSU 1114	*Mephitis mephitis* (striped skunk)	*L. canicola**	6	I & II	TNTC
				III	contaminated; leptospiral colonies also present

* Identity based on cross agglutinin-absorption tests with type strains.
** Based on cross-agglutination pattern against serotype-specific anti-Leptospira serums.
† Plate I, II, and III = inoculated with 0.1 ml. of 1:100, 1:1,000, and 1:10,000 dilutions of kidney suspension, respectively.
‡ TNTC = colonies too numerous to count.

were recovered in semisolid mediums but were not recovered by the agar plate method, and 13 strains were recovered by the agar plate method but were not recovered in the semisolid mediums employed (table 1).

Observations on 11 isolates obtained by the agar plate method are summarized (table 2). No concerted effort was made to classify, delineate, or correlate the colonial types. At least four variations of colonies were found; the growth in each instance appeared to be subsurface. Sometimes confluent or contiguous growth of leptospires occurred. The manner of inoculation, number of organisms present, and amount of fluid present on the surface of the agar possibly influenced the type of growth. On plates where the confluent growth occurred, the medium became hazy. Leptospires could easily be detected by dark-ground microscopy in pieces of agar removed from the plate and examined as described.

No difficulty was encountered in making subcultures from one agar plate to another. They were made by transferring a small piece of agar containing leptospires. A bacterologic loop was used to transfer and spread the inoculum. Transfers to Fletcher's semisolid medium and Stuart's liquid medium were made in the same manner and were usually successful.

Twelve strains of *L. pomona*, 5 strains of *L. canicola*, 2 strains of *L. icterohaemorrhagiae*, one strain of *L. ballum*, and one strain of *L. autumnalis* were identified by agglutination and absorption tests (table 1). The serogroup identity was determined for 34 additional strains; among these were 13 strains belonging to the *hyos* serogroup and 7 strains belonging to the *hebdomadis* serogroup.

Serotypes isolated by the agar plate medium where definite identity is known include *L. pomona*, *L. canicola*, *L. ballum*, and *L. icterohaemorrhagiae*. Two additional serotypes isolated were members of the *hebdomadis* and *hyos* serogroups.

Discussion

The agar plate method increased by 13 the total number of strains recovered. The number of primary isolations in this series was increased by eight. This appears to be due, at least in part, to the fact that contamination which precluded the possibility of successful isolation in semisolid mediums was overcome. Pure cultures of leptospires

could be obtained from plates even though some contaminating organisms were present. This was the case in the five isolations made from six attempts with the bovine kidneys. The sixth specimen was also grossly contaminated on the solid medium. In at least four instances, leptospiral organisms were recovered by the plate method; whereas, the semisolid mediums were negative, with no contamination present. However, 30 recoveries were made with semisolid medium when the companion plates were negative. The amount of inoculum was considerably greater in the semisolid mediums than on the plates; the inoculum for the plates was serially diluted so that the chance for overcoming contamination was greater. Had a greater amount of inoculum been used, the number of isolations may have been increased.

The number of colonies in some instances was proportional to the dilution factor. It appeared that, by using more refined methods, the number of organisms per gram of kidney could be estimated for some strains. Analysis of data pertaining to strain LSU 1013 (table 2) suggests that 64,000 organisms were present per milliliter of 10 per cent kidney suspension.

Another point of interest relates to the possible occurrence of mixed leptospiral infections under natural conditions. Studies from our laboratory suggest this possibility in cattle.[8] The agar plate method may prove of value in separating mixed infections present upon original isolation from the host animal.

SUMMARY

The agar plate method, used in conjunction with semisolid mediums, sufficiently increased the number of isolations of leptospires from the kidneys of naturally infected wild animals to warrant its inclusion on a routine basis. Data obtained also suggested its use for estimation of the number of leptospires present in the kidneys. Of 93 isolations, 50 were obtained from both agar plates and semisolid mediums. Thirty strains were recovered in semisolid mediums only; whereas, 13 strains were recovered by the agar plate method but were not recovered in semisolid mediums.

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SUMMARIO IN INTERLINGUA

Le Uso de Placas a Agar Como Adjuta in le Isolation de Leptospiras

Le methodo del placas a agar, usate in conjunction con medios semisolide, augmentava le numero del isolationes de leptospiras ab le renes de naturalmente inficite animales salvage de maniera sufficientemente marcate pro justificar le adoption del methodo como mesura routinari. Le datos obtenite etiam suggereva le utilitate del methodo in le estimation del numero de leptospiras presente in le renes. In un serie de 93 isolationes, 50 esseva obtenite tanto ab le placas a agar como etiam ab medios semisolide. Trenta racias esseva obtenite solmente in medios semisolide, durante que 13 esseva obtenite per le methodo a placas de agar sed non in medios semisolide.

Studies on Steroid Excretion in Calf Urine

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VARIOUS REPORTS have appeared in the literature concerning the determination of steroid metabolites in bovine urine.[3, 5, 10, 12, 14, 26, 30, 31] The methods employed in such studies were direct applications or minor modifications of procedures commonly used for the analysis of steroid metabolites in human urine.

The values obtained by these methods were recognized as being of doubtful significance, and further attempts were made to remove nonspecific chromogens by extensive purification and to establish a baseline level of steroid excretion in cattle.[11, 13, 15, 21] Additional experiments are reported in this paper which confirm the extreme difficulty encountered in establishing valid, meaningful, 17-ketosteroid and 17-hydroxycorticosteroid values for the urine of this species.

MATERIALS AND METHODS

Daily samples of urine from 6 ruminating calves were collected in individual receptacles with adequate precautions to preclude fecal contamination. The samples were preserved with the addition of toluene and were frozen immediately. Aliquots of individual frozen calf-urine samples, representing 5- to 8-hour collection periods, were thawed and subjected to one of the following purification procedures.

Hydrolysis of 17-Ketosteroids.—(1) Urine (calf 7) was extracted 3 times with 0.3 volumes of butanol. The residue was hydrolyzed by boiling for 10 minutes in 15-volumes per cent hydrochloric acid. (2) Bacterial β-glucuronidase (300 units/ml.) was incubated in buffered urine (pH 6.5) for 48 hours at 38 C. (3) Snail juice sulfatase (10 ml./liter) was incubated[9] in buffered urine (pH 5.4) (calves 1 and 3) for 48 hours at 38 C. (4) Hydrolysates of (2) and (3) were adjusted to pH 1.0 and were extracted 3 times with 0.5 volume of ethyl acetate. The urine residues were then subjected to additional hydrolysis by boiling for 10 minutes in 15 volumes per cent hydrochloric acid.

The ethyl acetate extracts of the hydrolysates were washed 3 times with 1/20 volume of 10 per cent sodium hydroxide, then 3 times with 1/20 volume of water. The resulting neutral extracts were treated overnight at room temperature with Girard's "T" reagent[29] to yield the ketonic and nonketonic steroid fractions.

Hydrolysis of 17-Hydroxycorticosteroids.—Aliquots of each calf urine sample, representing 11 to 16 hours of the daily collection period, were hydrolyzed with bacterial β-glucuronidase (300 units/ml.) at pH 6.5 for 48 hours at 38 C. The hydrolysates were adjusted to pH 1.0, and 10 per cent (w/v) sodium chloride solution was added prior to the ethyl acetate extraction.

Analyses of Extracts.—The ketonic fractions of the urine extracts were subjected to chromatography on a column containing an adsorbent substance* according to the method of Glenn and Nelson,[7, 23] followed by chromatography on paper in a toluene-propylene glycol[2] and a ligroin-propylene glycol system.[25]

Zimmermann assays for 17-ketosteroids were run according to the method of Holtorff and Koch,[16] and the Allen correction factor[1] was applied. The 17-hydroxycorticosteroid values were obtained by a slight modification of the Porter-Silber reaction.[28]

Administration of Cortisol-4-C^{14}.—A mixture of 5 μc. of cortisol-4-C^{14} in 100 mg. of carrier cortisol was injected into the jugular vein of a ruminating calf weighing 131 kg. Samples of blood were withdrawn prior to and 6, 25, 60, and 180 minutes following the injection of the steroid. Urine and fecal samples were collected for only the first 24 hours after injection of the steroid. The blood samples were frozen, thawed, and extracted in the cold with ethyl acetate.[19, 22]

Calf feces were thoroughly mixed with an alcoholic solution of thymol prior to freezing. The fecal samples were thawed and hydrolyzed by the addition of 20 per cent potassium hydroxide in 50 per cent methanol. The mixture was kept at room temperature for 48 hours with occasional stirring and then extracted repeatedly with chloroform. The combined chloroform extracts were thoroughly dried, and the lipids were removed by partition between 70 per cent methanol and petroleum ether.

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* Florisil, produced by Floridin Co., Inc., Tallahassee, Fla.

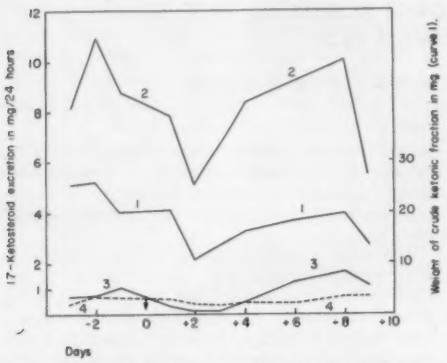

Fig. 1—17-ketosteroid excretion (milligrams per 24 hours) in butanol extracts of 8-hour samples of calf urine. (1) Crude weights of the ketonic fractions; (2) Zimmermann chromogen values of ketonic extracts prior to chromatography; (3) Zimmermann chromogen values of eluates following paper chromatography; (4) androgen values, in milligram equivalents of androsterone, obtained by chick comb bioassay of the ketonic extracts.

Aliquots of extracts containing radioactivity were transferred to aluminum planchets (31-mm. diameter) and were dried under a stream of nitrogen. Counts were determined on a Tracerlab"1000" Scaler with a flow-gas counting chamber.

RESULTS

Hot acid hydrolysis of the calf urine resulted in a two- to threefold increase in the weight of the crude ketonic fraction as compared with that obtained following snail juice sulfatase or β-glucuronidase hydrolysis. The crude ketonic weight was reduced by one third to one half with a corresponding reduction of the Zimmermann values after hot acid hydrolysis of butanol extracts of calf urine was accomplished. Zimmermann values obtained following β-glucuronidase hydrolysis were equal to those obtained following sulfatase hydrolysis. Greater difficulty was experienced with the extraction of sulfatase hydrolysates, due to the formation of heavy emulsions. An additional 20 per cent of Zimmermann chromogen was released with hot acid treatment following enzyme hydrolysis. There were low chromogen values following hydrolysis of butanol extracts with 20 per cent sodium hydroxide.[17]

The decrease in Zimmermann chromogen values with increasing purification of the ketonic fraction of butanol extracts of the urine of calf 7 is shown (fig. 1). The correspondence of the chromogen values with the crude ketonic weights is readily apparent. Curve 4 represents the androgen values in equivalents of androsterone obtained on the ketonic extracts, using the chick comb bioassay.

The Zimmermann chromogen values obtained following β-glucuronidase and hot acid hydrolysis of the urine of calf 3 are shown (fig. 2). The crude ketonic fractions gave an average 17-ketosteroid value of 21.5 mg./24 hours. Following chromatography of an absorbent column, the fractions eluted with chloroform gave a corresponding average 17-ketosteroid value of 13.3 mg./24 hours (fig. 2, curve 2). Further purification of these eluates by paper chromatography, using the ligroin-propylene glycol system,

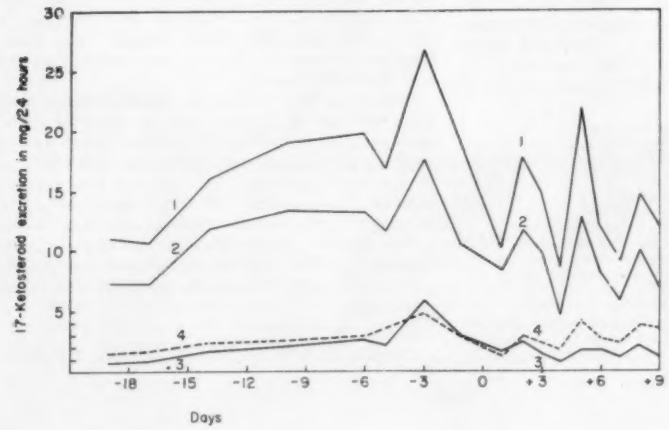

Fig. 2—Zimmermann chromogen values (milligrams per 24 hours) obtained by direct hydrolysis of calf urine. (1) Ketonic fraction before absorbent column chromatography; (2) C_{19} fraction obtained after column chromatography; (3) eluate of Zimmermann reacting zones following paper chromatography; (4) Allen correction factor applied to values represented in curve 1.

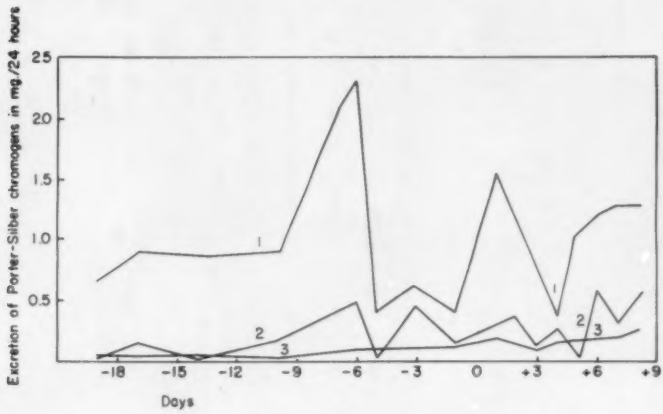

Fig. 3—Porter-Silber chromogen (milligrams per 24 hours) for β-glucuronidase-hydrolyzed calf urine. (1) Ketonic fraction before absorbent-column chromatography; (2) 17-hydroxycorticosteroid fraction obtained following column chromatography; (3) eluate of C_{21}-steroid zones obtained following paper chromatography in the toluene-propylene glycol system.

resulted in the detection of three Zimmermann reacting zones which migrated at a rate corresponding to that of $C_{19}O_2$ steroids. The average 17-ketosteroid value obtained on the eluted zones was 2.6 mg./24 hours. Curve 4 (fig. 2) represents the Zimmermann values obtained on the crude ketonic fractions by applying the Allen correction factor,[1] using the absorbances determined at 480, 520, and 560 mμ.

The 17-hydroxycorticosteroid excretion values, measured as Porter-Silber chromogens on β-glucuronidase hydrolyzed urine of calf 3, are shown (fig. 3). The proportionate decrease in Porter-Silber chromogens with increasing purification is similar to that observed for the Zimmermann chromogens. The crude ketonic extracts gave average Porter-Silber values of 0.9 mg. (range 0.4–2.3 mg.)/24 hours. Following adsorbent-column chromatography, the corticosteroid fraction [7] gave an average value of 0.3 mg./24 hours (range, 0.1–0.6 mg.). The eluates containing the corticosteroids were chromatographed on paper, using the toluene-propylene glycol system. An area which reacted with blue tetrazolium and migrated in the range of tetrahydrocortisol was eluted and assayed. The average Porter-Silber value following paper chromatography was 0.11 mg. (range, 0.08–0.26 mg.) per 24 hours (fig. 3, curve 3). Attempts to identify this material chemically as a steroid metabolite[s] were unsuccessful.

Administration of Cortisol-4-C^{14}.—Following the injection of 5 μc. of cortisol-4-C^{14} in 100 mg. of carrier cortisol into the jugular vein, only the first postinjection sample, collected after 6 minutes, contained any appreciable radioactivity (table 1). The blood extract of the 6-minute sample was chromatographed on paper in the toluene-propylene glycol system for 72 hours. A zone was detected corresponding to the cortisol standard, with ultraviolet absorption and a positive staining reaction to blue tetrazolium. The zone gave a total of 2,170 counts per minute. Further attempt at identification was not made.

TABLE 1—C^{14} Detected in Calf Blood Samples Following the Administration of 5 μc. of Hydrocortisone-4-C^{14}

Collection period (min.)	Peripheral blood C^{14}	
	Counts/20 ml.	μg.% F*
6	1,800	250
25	139	20
60	115	16
180	139	20

* Calculated on basis of administered hydrocortisone; specific activity = 35 counts/μg./min.

The total amount of radioactivity detected in the first 24-hour collection of urine amounted to only 1.5 per cent of the administered steroid (table 2). A single zone was detected with the blue tetrazolium reagent following paper chromatography of the urine extract in the toluene-propylene glycol system. The zone gave a total of 2,600 counts per minute and migrated at a rate corresponding to tetrahydrocortisol. Further attempt at identification was not made. The 24-hour collection of feces contained 4.0 per cent of the administered

radioactivity (table 3), the greater portion of which was excreted during the last 9 hours of the 24-hour period. The calf feces extract was purified by column chromatography and by paper chromatography. No definitive discrete zones could be detected with the usual steroid reacting reagents. Most of the radioactivity (100,0000 counts) remained in the most polar fractions, and further attempts at purification were not successful.

TABLE 2—Detection of C^{14} in Calf Urine Following the Administration of 5 μc. of Hydrocortisone-4-C^{14}

Urine fraction	Glucuronidase hydrolysis (counts/min.)	Acid hydrolysis (counts/min.)
Total neutral extract	54,000	51,400
Urine residue	11,000	.....
Alkali and water washes	4,200	.....
Ketonic fraction	31,000	28,700
Nonketonic fraction	22,000	14,700

DISCUSSION

The data obtained from the analyses of calf urine in this report indicate an extremely low titer of 17-ketosteroids and 17-hydroxysteroids. In addition, these results substantiate the conclusion that the earlier reports in the literature relating to steroid excretion in bovine urine are not meaningful. The magnitude of the Zimmermann and Porter-Silber chromogen values decreased in proportion to the purity of the urine extracts. The reliability of the data obtained by the procedures employed is at best tenuous, because a correlation in the fluctuations of the steroid values with the crude ketonic extract weights is similar to those obtained on extracts following rigorous purification. That nonsteroidal contaminating substances accompanied the steroid fraction throughout the course of purification was demonstrated by interpreting curves obtained on purified extracts. Typical chromogen absorption spectrums were seldom observed; however, application of the Allen correction factor in the Zimmermann determination did result in values that more closely approximated those obtained by bioassay.

When the pooling of the ketonic extracts was followed by extensive paper chromatography, steroid reacting zones were seen, but material of sufficient purity could not be isolated for positive identification. Marker and co-workers [17,18] isolated androsterone and dehydroepiandrosterone from large pools of bovine urine in a concentration range of 0.002 to 0.05 mg. per liter. Even with compensation for losses due to the crude isolation procedures employed at that time, it was evident that relatively small amounts of these compounds were present in the urine. The chemical analysis for 17-ketosteroids at this concentration level would still be feasible if it were not for the presence of contaminating substances which contribute to the final chromogen values. These nonsteroidal contaminants have been identified by Holtz [12,13] and substantiated by Mixner *et al.*[21] as ionone derivatives.

The nature of the Porter-Silber chromogens obtained in this report leads to conclusions similar to those discussed for the Zimmermann chromogens. The low Porter-Silber chromogen value of bovine urine was reported previously by Holm and Firch [10] and by Holcombe.[15] In the latter study, Holcombe compared the values obtained by various assay procedures available for corticosteroid analyses and concluded that the procedure based on the Heard and Sobel [8] reaction gave results that correlated best with the steroid hormone status of the animal. The chromogens obtained by this procedure, which is one of the least specific of the reactions tested, were called "reducing corticoids." Until the nature of these "reducing corticoids" has been identified and their relationship to steroidal material is established, any interpretation of the results obtained by this procedure may be seriously questioned.

TABLE 3—Excretion of C^{14} in Calf Feces Following the Administration of 5μc. of Hydrocortisone-4-C^{14}

Collection period (hr.)	Fecal C^{14} excretion: Total counts/collection* (counts/min.)	Fecal C^{14} excretion: Counts/min./Gm.
0–6	0	0
7–15	22,000	12
16–21	71,400	40
22–24	57,000	72

* Total petroleum ether washes contained 6,000 counts per minute.

Following the administration of cortisol-4-C^{14}, only 1.5 per cent of the radioactivity could be detected in the first 24-hour urine

collection. This compares well with the detection of 0.18 to 0.77 per cent of 17-hydroxysteroids, reported by Holm and Firch,[10] following the administration of cortisone. However, Holcombe [15] reported a 22 to 40 per cent recovery of administered cortisone as "reducing corticoids."

Evidence for the role of the bile as an important factor in the metabolism and excretion of C_{18}, C_{19}, and C_{21} steroids in the cow has accumulated.[24] The androgen content of feces of lactating cows has been given considerable attention [6] and, more recently, C_{19} steroids have been isolated from cow feces by Miller *et al.*[20] In this study, 4.0 per cent of the radioactivity administered as cortisol-4-C^{14} was detected in the first 24-hour fecal sample, with the major portion being excreted in the last 9 hours. Previously, it was shown that in man fecal excretion of administered C^{14}-cholesterol was delayed 48 to 72 hours.[32] If a comparable lag in corticosteroid excretion occurs in the cow, it would have resulted in greater amounts of radioactivity being detected in the second and third days samples of feces. However, the possibility of a delayed excretion of metabolites in the urine cannot be ruled out.

The rapid decrease in the circulating blood level of administered cortisol-4-C^{14} from 250 μg. per cent at 6 minutes to less than 20 μg. per cent at 25 minutes is in agreement with the previous findings of Holm and Firch [11] and of Mixner and Robertson.[22, 27] The latter workers obtained a half-life of 9 minutes for cortisol under conditions similar to those employed in this study.

The amounts of corticosteroids excreted in the urine of the calf are of a much lower order of magnitude than would be expected, considering the amounts of steroids secreted by the perfused, intact, calf adrenal gland.[33] Confirmation of the low rate of excretion of corticosteroids in the urine of this species was obtained by the administration of cortisol-4-C^{14}. Since only a minute fraction of the steroid metabolites is excreted in the urine in the first 24 hours, and since difficulties are encountered in removing nonspecific chromogens, it is apparent that any interpretation of urinary steroid excretion as a reflection of the status of the steroid hormones in this species would be particularly hazardous.

SUMMARY

Estimations of 17-ketosteroids and 17-hydroxysteroids in urine samples of 6 ruminating calves were attempted, using various hydrolytic and purification procedures. The Zimmermann and Porter-Silber chromogen values obtained were of doubtful significance due to the apparently low levels of excreted steroid metabolites and the failure of the procedures employed to remove contaminating substances which interfered with the proper development of the expected chromogens. Because of the doubtful nature of the analytical results, and from the evidence obtained following the administration of hydrocortisone-C^{14}, it must be concluded that, with currently available methods, the analysis of urinary steroids is not suitable to assess the status of the steroid hormones in the bovine species.

Of the cortisol-4-C^{14} administered to 1 calf, 1.5 per cent was detected in the urine and 4.0 per cent was detected in the feces during the first 24 hours.

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SUMMARIO IN INTERLINGUA

Studios in Re le Excretion de Steroides in le Urina del Vitello

Estimationes del nivellos de 17-cetosteroides e 17-hydroxysteroides in specimens del urina de ruminante vitellos esseva essayate con le uso de varie technicas de hydrolyse e de purification. Le valores assi obtenite pro chromogeno de Zimmermann e Porter-Silber esseva de signification dubitose in consequentia del apparentemente basse nivellos de excernite metabolitos steroidic e del facto que le technicas usate non succedeva a eliminar le substantias contaminatori que disturbava le efficace disveloppamento del expectate chromogenos. A causa del character dubitose del resultatos analytic e super le base del observationes effectuate post le administration de hydrocortisona a C^{14}, le conclusion se impone que le currentemente disponibile methodos non suffice a render le analyse de steroides urinari adequate como methodo pro le evalutation del stato del hormones steroidic in le specie bovin.

Ab le cortisol-4-C^{14} que esseva administrate a un vitello, 1,5 pro cento esseva detegite in le urina e 4,0 pro cento esseva detegite in le feces del prime 24 horas.

Studies on the Erythrocyte Sedimentation Rate in Domestic Animals. VII. Rouleau Formation

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BLOOD obtained from pregnant women and from persons affected with infectious diseases is characterized by rapid sedimentation of erythrocytes. This phenomenon is accompanied by arrangement of the erythrocytes into rouleaux, having the appearance of stacked coins. When the phenomenon is marked, the rouleaux tend to clump together as in so-called pseudohemagglutination.

There are some reports on rouleau formation of equine erythrocytes. Gulliver [26] pointed out that the rapid sedimentation of horse blood is due to a physiologically pronounced aggregation of the blood corpuscles. Haan [26] also reported that rapid sedimentation of horse blood is caused by intense rouleau formation, and that it can be slowed by adding physiologic sodium chloride solution.

The influence of shape, size, and specific gravity of the blood corpuscles on the sedimentation rate is negligible as compared to that of rouleau formation.[26] According to Dukes,[6] the only factor of importance is the degree of erythrocyte agglutination, and it is certain that the plasma protein markedly influences this factor. However, horse erythrocyte sedimentation is rapid, even in ox, goat, and sheep plasma; sedimentation of ox, goat, and sheep erythrocytes is slow in horse plasma. These findings indicate that the plasma is not the only factor determining the rapid sedimentation of horse erythrocytes. Thygesen [26] stated that the aggregation is affected partly by the blood corpuscle and partly by the properties of plasma.

Sesaki [22] reported that in horses, pigs, and dogs the sedimentation rate decreases in proportion to the degree of dilution of plasma; this was attributed to a reduction of the rouleau-promoting factor in the plasma rather than to the decrease of specific gravity and viscosity.

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MATERIALS AND METHODS

The experiments were carried out with blood from both diseased and clinically healthy animals, including 12 horses, 6 cattle, 5 sheep, 7 goats, 5 swine, 14 dogs, 6 rabbits, and 5 chickens.

The Westergren method was used to measure the erythrocyte sedimentation rate (e.s.r.), and a drop of this blood was examined microscopically.

To investigate the relationship between rouleau formation and the dilution of plasma, blood subjected to the Westergren method was centrifuged and the plasma was diluted with 3.8 per cent Na citrate solution. The ratios of plasma to citrate solution were 4:1, 3:2, 2:3, and 1:4. The erythrocytes obtained were then added to these diluted plasma samples.

Plasma-exchange experiments were made with heat-inactivated plasma (30 min. at 56 C.) and blood corpuscles of the different species. Washed erythrocytes, 0.5 ml., were added to 1.5 ml. of inactivated heterogeneous plasma and the suspension was incubated at 37 C. for 15 minutes. After centrifugation, the erythrocytes in the heterogeneous plasma were examined microscopically. If they were clumped together, the suspension was diluted with 3 volumes of physiologic saline solution to distinguish rouleau formation from true agglutination; rouleau formation is abolished by this procedure.

RESULTS

Rouleau Formation in Normal Animals.—Long rouleaux were always observed in horse blood, and these were clumped together as in pseudohemagglutination. In swine blood, short rouleau formations were observed. There was no rouleau formation in the blood of ruminants, dogs, rabbits, and chickens.

Rouleau Formation in Diseased Animals.—Intense rouleau formation in horse blood and moderate rouleau formation in swine blood, with an increased sedimentation rate (11 to 25 mm. in 2 hours), were always observed. In blood from dogs with various diseases, and from pregnant bitches, rouleaux were always observed. In blood from a rabbit given an injection of Sr^{90}, rouleau formation also occurred. There was no rouleau formation in blood from diseased ruminants.

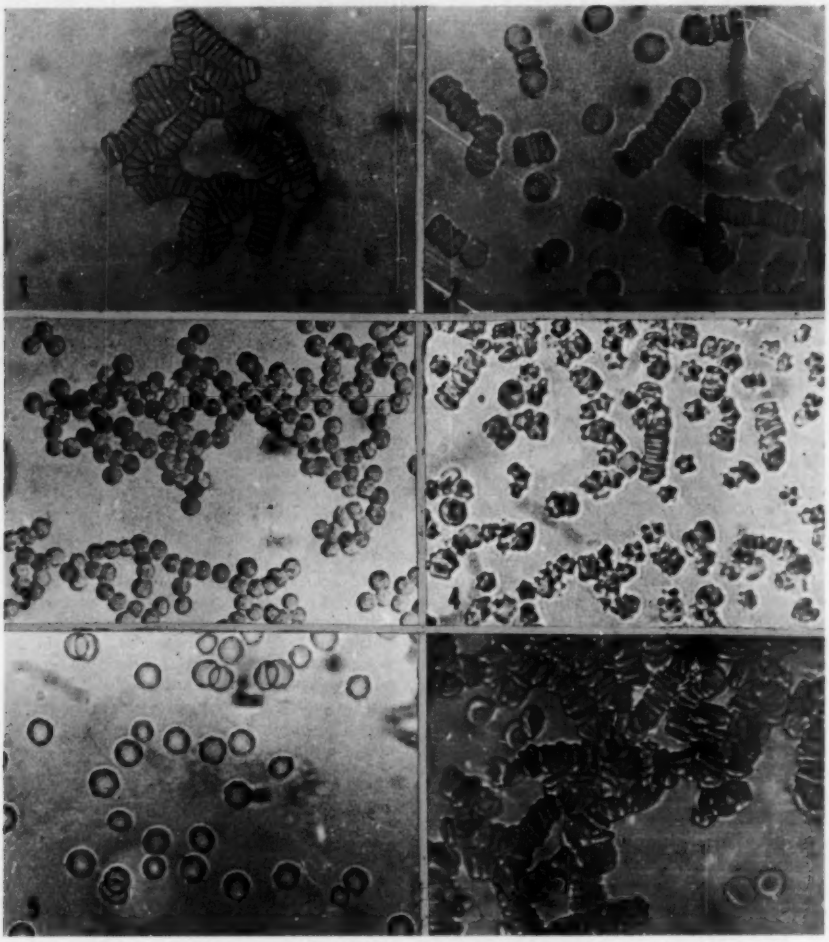

Fig. 1—Moderate rouleau formation (++) of horse erythrocytes in homologous plasma diluted 1.5 times.

Fig. 2—Weak rouleau formation (+) of horse erythrocytes in homologous plasma, diluted 2 times.

Fig. 3—Weakly agglutinated horse erythrocytes in cattle plasma.

Fig. 4—Horse erythrocytes in chicken plasma; weak rouleau formation.

Fig. 5—Erythrocytes (normal dog) in homologous plasma; no rouleau formation.

Fig. 6—Intense rouleau formation (+++) in blood from dog 95 with dirofilariasis. The erythrocyte sedimentation rate was 19 mm./hr. and 54 mm./2 hr.

Plasma Dilution and Rouleau Formation.—The degree of rouleau formation was, in general, inversely proportional to the degree of plasma dilution (fig. 1, 2). Diluting the plasma with 3.8 per cent Na citrate solution (3 times for normal horse blood, 1.5 times for normal swine blood, 3 times for blood from sick dogs) resolved the rouleaux into single blood corpuscles.

Influence of Erythrocyte Dilution on Rouleau Formation.—There was no apparent correlation between the dilution of erythrocytes and rouleau formation.

Rouleau Formation in Heterogeneous

Plasma.—Erythrocytes of horses were, in general, agglutinated in cattle plasma; clear rouleau formation was usually, but not always, observed. The same results were obtained with goat and sheep plasma. Obscure rouleau formation was observed in swine plasma, but rouleaux were not observed in dog plasma. Short rouleaux were always observed in chicken plasma.

Rouleau formation of swine erythrocytes was generally obscure in horse plasma, but occasionally rouleaux formed clearly. Rouleau formation was not observed in ruminant, dog, rabbit, and chicken plasma.

Intense or weak rouleau formation of dog erythrocytes was always observed in horse, swine, and cattle plasma, but not always in goat and sheep plasma. Rouleaux were not formed in rabbit and chicken plasma.

There was no rouleau formation of ruminant, rabbit, and chicken erythrocytes in heterogeneous plasma.

Relationship Between Rouleau Formation and Erythrocyte Sedimentation Rate.—As shown (table 1), intense rouleau formation corresponded with an increased sedimentation rate.

TABLE 1—The Relationship Between the Degree of Rouleau Formation and the Erythrocyte Sedimentation Rate in Dogs

Dog No.	e.s.r. in 2 hr. (mm.)	Degree of rouleau formation*	Condition
81	2	—	Normal
85	7	—	Normal
77	7	—	Normal
82	8	+	5th day after parturition
88	20	++	Distemper
82	25	+++	Pregnant
90	26	+++	Distemper
87	52	+++	Distemper
95	54	+++	Dirofilariosis
89	70	+++	Distemper
91	70	+++	Distemper
83	85	+++	Poisoning
79	100	+++	Splenectomy
78	105	+++	Splenectomy
77	135	+++	Bone fracture

* — = no rouleau formation; + = weak rouleau formation (several erythrocytes clumped together); ++ = moderate rouleau formation (rouleaux clumped together); +++ = intense rouleau formation (rouleaux markedly clumped together).

DISCUSSION

There is a considerable interspecies variation in the erythrocyte sedimentation rate; *i.e.*, the erythrocytes of horses settle quickly, and those of ruminants settle slowly.

According to Fahraeus,[26] a greater tendency toward rouleau formation accompanies a more rapid sedimentation in human blood. As shown (table 1), the erythrocyte sedimentation rate of diseased dogs is also roughly proportional to the degree of rouleau formation.

Sesaki [22] reported that the sedimentation rates of the horse, the pig, and the dog were decreased in parallel with the degree of the dilution of plasma, suggesting that the decreased sedimentation rate is a result of a reduction of the rouleau-promoting factor in the plasma.

As already stated, the degree of rouleau formation is, in general, inversely proportional to the dilution of horse and swine plasma. Diluting the plasma with 3.8 per cent Na citrate solution (3 times for normal horse blood, 1.5 times for normal swine blood) resolved the rouleaux into single blood corpuscles. Thus, rouleau formation is most intense in horse blood, and the degree of rouleau formation is responsible for the increased sedimentation rate of horse, swine, and dog erythrocytes.

Pertaining to rouleaux formation, there are such theories as surface tension, electric charge, hydrogen, and Thygesen's theory. Thygesen's theory [26] is as follows: Since the ionic atmosphere has a total charge of the same order of magnitude as the charge of the central ion, although of an opposite sign, an ion and its atmosphere represents, from outside, an electrically neutral formation. Due to this electric shielding, 2 equally charged central ions can approach one another without repulsion until deformation of the ionic atmosphere begins. In the case of erythrocytes, the forces are assumed to be electrostatic Coulomb forces which, due to the tendency of Debye-Milner's ionic atmosphere to attain central symmetry, compel the equally charged corpuscles to aggregate.

Hartmann [13] found, *in vitro*, that albumin has a slowing effect on the sedimentation rate, while globulins in high concentration (above 5%) and fibrinogen in concentrations over 0.5 per cent increase the sedimentation rate. Ferguson [7] reported that the relatively high fibrin content of normal bovine serum may account for the slow sedimentation rate. Fletcher [8] stated that the administration of cortisone or adrenocorticotrophic hormone (ACTH) in amounts sufficient to induce a clinical re-

mission usually results in a dramatic decrease in both the sedimentation rate and the plasma fibrinogen, often to normal levels. Changes in the serum globulin occur more slowly and with practically no relationship to the sedimentation rate. Hunt and Trew [15] pointed out that there was a linear correlation between sedimentation rate and plasma fibrinogen levels. Raul Perez Castrillo [20] described the increase in erythrocyte sedimentation rate as proportional to the *gamma* globulin added; photomicrographs depicted agglutination of erythrocytes by *gamma* globulin. Hasché,[14] however, reported that the sedimentation rate and plasma fibrinogen content showed no interdependence. Bielski and Karon [3] also reported that there was no strict correlation between the blood sedimentation rate and the blood fibrinogen level, but that in acute inflammatory states an increase of this rate seems nevertheless to be caused by increased fibrinogen. The acceleration of blood sedimentation observed in chronic diseases is probably due to some disturbances in the globulin fraction and to concomitant erythropenia.

Many investigators, however, believe that the sedimentation rate is, in general, approximately proportional to the amount of fibrinogen and globulin and inversely proportional to the amount of albumin present. In horses and cattle, normal blood-albumin levels are 3.25 Gm./100 ml. and 3.63 Gm./100 ml., respectively.[6] If the albumin has a slowing effect, it could be responsible for the slower sedimentation rate of cattle blood than that of horse blood. Accordingly, the sedimentation rate of sheep blood would be expected to be faster than that of horse blood, because the amount of albumin in sheep blood is 3.07 Gm./100 ml.[6] However, this is contrary to fact. The globulin level of horse plasma is higher than that of cattle [6]; if erythrocyte sedimentation rate is in positive correlation to total globulin, cattle erythrocytes will sediment more rapidly than those of horses. This also is contrary to fact. Therefore, no absolute correlation is found between species variation in erythrocyte sedimentation rate and any of the plasma protein fractions.

White and Monaghan [27] investigated the erythrocyte sedimentation rate, rouleau formation, and electrophoretic mobility of dog, horse, cat, pig, cattle, and rabbit erythrocytes in 1 per cent gelatin at pH 7.4. They stated that at higher mobilities there is greater aggregation. These findings indicate that the free surface energy at the cell-medium interface is the determining factor.

Ponder [19] examined the conditions governing the rate of rouleau formation and found that the sticky surface of the corpuscle seems to play a more important part than the potential difference between cells and suspending fluid.

Rouleau formation of horse erythrocytes always occurs in chicken plasma, in which chicken erythrocytes do not form rouleaux. However, there is no rouleau formation of ruminant and chicken erythrocytes in horse plasma. These findings indicate that rouleau formation is affected partly by the blood corpuscles. From the results of these experiments, the author postulates that the red blood cell factor is predominant in determining differences in rouleau formation of the various species.

SUMMARY

Rouleau formation was analyzed in blood from both diseased and clinically healthy animals, including 12 horses, 6 cattle, 5 sheep, 7 goats, 5 swine, 14 dogs, 6 rabbits, and 5 chickens.

The rouleau formation was always observed in citrated blood of normal horses and swine. In horse blood, especially, rouleaux were clear, long, and tended to clump together as in pseudohemagglutination. There was no rouleau formation in the blood of normal dogs, ruminants, rabbits, and chickens.

Rouleau formation was observed in blood from diseased horses, dogs, and rabbits, but not in blood from diseased ruminants.

The erythrocytes of horses, swine, and dogs formed rouleaux in heterogeneous plasma, but those of ruminants and chickens did not.

Erythrocyte sedimentation rates were roughly proportional to the degree of rouleau formation, and the length of rouleaux was shortened as plasma was diluted.

Species variation in the rouleau formation of blood from normal animals is probably caused by properties of erythrocytes; whereas, the increase of rouleau formation in diseased animals may be attributed to the changes in the plasma.

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SUMMARIO IN INTERLINGUA

Studios in Re le Sedimentation Erythrocytic in Animales Domestic. VII. Formation de Rouleaus

Le formation de rouleaus esseva analysate in sanguines ab animales morbide e ab animales clinicamente normal. Le donatores includeva 12 cavallos, 6 pecias de bestial, 5 oves, 7 capras, 5 porcos, 14 canes, 6 conilios, e 5 gallinas.

Le formation de rouleaus esseva semper observate in citrate specimens de sanguine ab cavallos e porcos normal. In le specimens equin, in particular, le rouleaus esseva clar e longe e tendeva a aggregar se como in le phenomeno de pseudohemagglutination. Esseva trovate nulle formation de rouleaus in le sanguine de canes, ruminantes, conilios, e gallinas normal.

Le formation de rouleaus esseva observate in le sanguine de morbide cavallos, canes, e conilios sed non in le sanguine de morbide ruminantes.

Le erythrocytos de cavallos, porcos, e canes formava rouleaus in plasma heterogenee. Le erythrocytos de ruminantes e gallinas non monstrava iste phenomeno.

Le rapiditate del sedimentation erythrocytic esseva grossiermente proportional al grado del formation de rouleaus, e le longor del rouleaus esseva reducite quando le plasma esseva diluite.

In le caso de sanguines ab animales normal, differentias secundo le species in le formation de rouleaus es probabilemente causate per proprietates del erythrocytos. Le augmento del formation de rouleaus in animales morbide es probabilemente attribuibile a alterationes in le plasma.

A New Method for the Primary Evaluation of Anti-Coccidial Activity

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THE BASIC PURPOSE of a laboratory program directed toward the development of chemotherapeutic drugs is to detect biological activity, which must then be confirmed and defined by extensive clinical or field trials, or both. The usual laboratory appraisal of chemotherapeutic agents for coccidiostatic activity follows a standard testing procedure that involves the mixing of drugs at different levels in feed.* The medicated feeds are introduced at least one day prior to exposure to coccidia, and are continued ad libitum through the critical period of infection.

Standard 5-deck battery brooders, usually used in these tests, require a floor space of 3 by 5 ft. and impose space restrictions on the testing of chemicals. To evaluate a large number of experimental compounds, many brooders and feed mixers are required in a spacious working area.

This is a report of a direct-dosing technique developed for the primary laboratory evaluation of chemicals for anti-coccidial activity. The method was developed to carry out a large-scale anti-coccidial program in a limited area at a cost below that of the standard drug-diet method. It affords considerable saving in time, space and drug requirements.

MATERIALS AND METHODS

Eight-day-old New Hampshire Red chickens (60 to 70 Gm.), which are highly susceptible to *Eimeria tenella*, were used in these experiments. The *E. tenella* was selected because it is ordinarily more sensitive to drug action than other species of coccidia.

During the second week of life, a chicken will consume an average of 20 Gm. of feed a day. From this it can be calculated that 20 mg. of drug per chick per day is equivalent to 0.1 per cent in the feed; the lower level of 10 mg. is equal to 0.05 per cent in the ration. In the initial experiment with known coccidiostats, both levels were evaluated on a 5-day treatment schedule. Test drugs were administered directly into the crop in a volume of 0.25 ml. with a 2-inch, blunt-end, 18-gauge needle attached to a 2-ml. tuberculin syringe. The *E. tenella* inoculum was introduced in the same manner.

On the first day of an experiment, the drug was administered in the morning; on the second day, it was given in the morning and the chickens were inoculated with 50,000 sporulated oocysts of *E. tenella* during the afternoon. On the third, fourth, and fifth days, the drug was given to complete the five-day schedule. Test animals were held until the eighth day, when they were necropsied and examined for evidence of infection. The efficacy of a drug was judged by the prevention of mortality and by a comparison of the pathologic index with that of the unmedicated infected controls. At necropsy, the degree of pathologic involvement was expressed as the average degree of infection, based on the following scheme: 0 = no lesions in cecum; 1 = slight lesions; 2 =

Fig. 1—Animal rack with a 420-chicken capacity.

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TABLE 1—Activity of Selected Coccidiostats Against Eimeria tenella Using the Five-Day Direct-Dosing Method

Coccidiostat	Mg./chick	Mortality (%)	Average degree of infection
Nicarbazin	20	0	1.2
	10	0	1.5
Infected controls	....	70	3.7
Sulfaquinoxaline	20	0	2.0
	10	0	2.2
Infected controls	....	50	3.5
3,5-dinitrobenzamide	20	0	2.4
	10	0	2.8
Infected controls	....	50	3.5
Nitrophenide	20	0	2.8
	10	0	3.0
Infected controls	....	50	3.5
Trithiadol	20	20	2.2
	10	20	3.4
Infected controls	....	100	4.0
Unistat	20	0	1.0
	10	20	2.0
Infected controls	....	100	4.0
Glycamide	20	0	0.8
	10	0	1.2
Infected controls	....	70	3.7
Zoalene	10	0	2.2
Infected controls	....	70	3.7

moderate lesions; 3 = severe lesions; and 4 = death due to infection.

The results with control drugs were satisfactory because reproducible anti-coccidial activity was demonstrated. Compounds found to be active under these conditions were further evaluated by the drug-diet method against *E. tenella* and other important species of coccidia.

To facilitate the handling of a large number of chickens, an animal rack was designed that occupied a small floor area. It is 28 inches wide at the wheel base, 6 ft. high, and 7 ft. long (fig. 1). Each section (cage) is 10 inches wide, 8 inches high, and 18 inches deep. The individual sections conveniently house up to 10 young chickens; total rack capacity is 420 birds.*

Actual operation of the direct dosing method is illustrated (fig. 2). Using the 2 racks shown, a total of 84 separate tests can be handled at one time in an area of only 35 square feet. The whole operation can be carried on by one technician with 2 experiments running concurrently.

Results and Discussion

For a new method to be useful, it is necessary to determine that known coccidiostats are active under the conditions of the system. Commercial materials were active with the direct dosing method against a highly virulent strain of *E. tenella* (table 1). All coccidiostats prevented mortality, and a suppression of infection was evident in all cases. Nicarbazin has been used as the standard in approximately 75 experiments. The results with this chemical complex have not varied significantly from the data shown (table 1).

Experience with this method indicated that compounds capable of preventing mortality have anti-coccidial activity. Under this new system, the fifth treatment was given 72 hours after inoculation with the culture. This time is a critical stage in the development of infection.

The figures presented for the commercial coccidiostats are from experiments in which these agents have been used as working standards. Test compounds that had activity comparable to that of the working standards used in a particular trial were then evaluated further by the use of the conventional drug-diet method.

It has been shown that the new method demonstrates the activity of several commercial coccidiostats. Nitrofuran compounds are the exception, because they are toxic upon direct-dosing at the test levels of 20 mg. and 10 mg. per chicken, and inactive at doses of 5 mg. per chicken and lower.

To demonstrate the sensitivity of this new technique, the activity of several test compounds evaluated at 10 mg. per chicken is presented (table 2). All compounds evaluated by the direct-dosing method had

TABLE 2—Comparative Anti-Coccidial Activity of Selected Test Compounds Using Direct-Dosing and Diet Feed Methods

Compound	Levels	Mortality	Average degree of infection
DIRECT-DOSING METHOD			
2656	10 mg.*	0/5	0.8
2657	10 mg.	0/5	1.0
Nicarbazin	10 mg.	0/5	1.5
Infected controls		7/10	3.7
2021	10 mg.	0/5	2.2
2022	10 mg.	0/5	1.3
2023	10 mg.	0/5	1.0
Nicarbazin	10 mg.	0/5	1.4
Infected controls		8/10	3.8
2211	10 mg.	0/5	1.0
Nicarbazin	10 mg.	0/5	1.6
Infected controls		4/5	3.8
DRUG-DIET METHOD			
2656	0.025%**	12/20	3.6
2657	0.025%	14/20	3.7
Nicarbazin	0.010%	0/20	1.4
Infected controls		8/10	3.8
2021	0.025%	14/20	3.7
2022	0.025%	14/20	3.7
2023	0.025%	12/20	3.6
Nicarbazin	0.010%	0/20	1.4
Infected controls		5/10	3.5
2211	0.025%	8/20	3.0
Nicarbazin	0.010%	0/20	1.4
Infected controls		7/10	3.7

* Dosage per chicken. ** Per cent of compound in the ration.

* Constructed by the Dixie Cage Co., Falls Church, Va.

Fig. 2—The direct-dosing method in operation.

significant activity when compared to nicarbazin; these same compounds were ineffective at 0.025 per cent by the drug-diet method (table 2). Nicarbazin had good activity by either method.

Even though the chemicals cited were not active when incorporated in the ration, such information is valuable to the chemist, because these compounds could logically serve as the basis of activity leads in the synthesis of more effective members of a series. The drug-diet method would not have permitted similar detection of activity. This new technique is therefore a valid means for economically assessing the efficacy of a large number of potential coccidiostats.

SUMMARY

This communication describes a new direct-dosing technique in chickens for the primary laboratory evaluation of chemicals for anti-coccidial activity. The method was developed to carry out a large-scale anti-coccidial program in a limited space, at a cost below that of the standard drug-diet test. The direct-dosing method saves time, space, and drugs.

SUMMARIO IN INTERLINGUA

Un Nove Methodo pro le Evalutation Primari de Activitate Anti-Coccidial

Iste communication describe un nove technica pro le medication directe de gallinas in le evalutation laboratorial primari del activitate anti-coccidial de agentes chimotherapeutic. Le methodo esseva disveloppate pro render possibile le execution de un programma anti-coccidial in massa in spatios restringite e a un costo infra illo del test standard con dietas medicate. Le methodo del medication directe es economic in tempore, spatio, e drogas, e illo es plus sensibile que le technica conventional.

An *in Vitro* Study of Extracted Antigens of *Vibrio fetus* Isolates

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AN UNDERSTANDING of the antigenic composition of *Vibrio fetus* is basic to the production of an effective immunizing agent against vibriosis and to the development of a useful diagnostic reagent. The diverse antigenic structure of various *V. fetus* isolates has been demonstrated by the unreliability of the serum-agglutination [3, 6] and cervicovaginal mucus agglutination tests.[2, 6] These observations of unreliability of existing immunologic tests have been substantiated by data obtained from antigen studies by a number of investigators.[1, 4, 5, 7, 13, 16]

Antigens have been extracted from *V. fetus* cells by chemical fractionation by Gallut,[5] Mitscherlich and Liess,[8] and Ristic and Brandly.[11] Gallut [5] utilized a 95 per cent phenol extraction and succeeded in separating a carbohydrate fraction and a protein fraction which was specific for 9 of the 10 Vibrio isolates studied. Mitscherlich and Liess [8] extracted an antigenic substance with phenol from catalase-positive and catalase-negative *V. fetus* cells. On the basis of complement-fixation titers obtained against various *V. fetus* antiserums, the authors concluded that the catalase-positive Vibrio possessed 2 antigenic fractions, designated antigen (1) and antigen (2). Antigen (3) was postulated as characteristic of the catalase-negative vibrios, as no cross reaction existed between the antigens of catalase-positive vibrios and antiserum prepared against catalase-negative vibrios. More recently, Ristic and Brandly [11] isolated a heat-stable soluble (HS) antigen from a smooth form of *V. fetus*. Cross-hemagglutination reactions between erythrocytes sensitized with HS antigen and specific antiserums of strains from various origins and serogroups showed that this polysaccharide was possessed by many, but not all, *V. fetus* strains.[12]

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From the Department of Pathology and Bacteriology and the Experiment Station, Colorado State University, Fort Collins. This study was an integral part of regional project, W-27, Vibriosis of Sheep. The present address of the senior author is the Division of Veterinary Science, College of Agriculture, University of Wyoming, Laramie.

This work is a portion of the dissertation submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree (bacteriology) at Colorado State University, 1959.

MATERIALS AND METHODS

Cultures.—The ovine (O) and bovine (B) cultures used in this study were from isolates from aborted fetuses at the Colorado State University diagnostic laboratory. The human (H) cultures were received from Dr. E. O. King, Communicable Disease Center, Chamblee, Georgia.

Saline Extraction.—Shaker-grown [14] cells of each *V. fetus* culture were harvested from 40 liters of brucella broth (Albimi) with the Sharples 1A centrifuge. The cells were suspended in 100 ml. of 0.9 per cent NaCl solution in a 400-ml. beaker, stirred continuously for 8 hours at 4 C., and centrifuged at 0 C. The supernatant fluid was dialyzed against running tap water (10 C.) for 24 hours, against 3 changes of distilled water (4 C.) for an additional 24 hours, and pervaporated to one-third volume. The saline extraction was repeated 2 additional times and the fractions were combined. The combined extracts were lyophilized, designated as saline fractions S, and stored at 4 C. in plastic-stoppered vials in a desiccator jar containing $CaCl_2$. The cells were saved for the 2 per cent phenol extraction.[12]

Phenol Extraction.—The saline-extracted cells were suspended in 100 ml. of 2 per cent phenol in a 400-ml. beaker and stirred continuously for 8 hours at 4 C. The cell suspension was centrifuged as above and the supernatant fluid dialyzed 24 hours against running tap water and an additional 24 hours against 3 changes of distilled water. After pervaporation to one-third volume, it was lyophilized, designated as phenol fraction P, and stored at 4 C. in a plastic-stoppered vial in a desiccator jar with $CaCl_2$. The cells were discarded.

Immunologic Procedures.—A 1:500 distilled water solution of each saline and phenol fraction of 8 Vibrio isolates (O-3, O-5, O-15, O-21, B-8, H-33, H-38, and H-40) was tested against *V. fetus* whole-cell rabbit antiserums for these 8 isolates plus the antiserums of 2 other isolates (B-6 and B-7), using the Oakley and Fulthorpe [9] and Ouchterlony [10] precipitin techniques.

RESULTS AND DISCUSSION

Using the Oakley-Fulthorpe precipitin technique, the following common-antigen groups appeared (table 1): group I, *V.*

TABLE 1—Oakley-Fulthorpe Precipitin Reactions of Saline (S) and Phenol (P) Extracted Antigens of Vibrio fetus Isolates against Whole-Cell Rabbit Antiserums

Antigen	Whole cell antiserums									
	O-3	H-38	O-5	O-15	O-21	B-6	B-7	B-8	H-33	H-40
O-3S	+	+	0	0	+	+	+	+	+	+
O-3P	+	+	0	0	+	+	+	+	+	+
H-38S	+	+	0	0	+	+	+	+	+	+
H-38P	+	+	0	0	+	+	+	+	+	+
O-5S	0	0	+	+	+	+	+	+	+	+
O-5P	0	0	+	+	+	+	+	+	+	+
O-15S	0	0	+	+	+	+	+	+	+	+
O-15P	±	0	+	+	+	+	+	+	+	+
O-21S	+	+	+	+	+	+	+	+	+	+
O-21P	+	+	+	+	+	+	+	+	+	+
B-8S	+	+	+	+	+	+	+	+	+	+
B-8P	+	+	+	+	+	+	+	+	+	+
H-33S	+	+	±	±	+	+	+	+	+	+
H-33P	±	+	+	±	+	+	+	+	+	+
H-40S	+	+	+	+	+	+	+	+	+	+
H-40P	+	+	+	+	+	+	+	+	+	+

fetus O-3 and H-38; group II, *V. fetus* O-5 and O-15; and group III, *V. fetus* O-21, B-6, B-7, B-8, H-33, and H-40.

Group I was distinct from group II because the saline and phenol fractions of the *V. fetus* isolates in group I did not react with the antiserums prepared against the organisms in group II. Except for a very faint precipitate which appeared after 15 days with the O-15 phenol fraction against the O-3 antiserum, consistent results were obtained in the reciprocal test with *V. fetus* whole-cell rabbit antiserums. Group III was distinct from groups I and II in that the saline and phenol fractions reacted with antiserums prepared against all cultures.

No significant differences were observed between the phenol and saline extracts used as antigens.

Study of the diffusion patterns produced by the same phenol and saline extracts in Ouchterlony agar diffusion plates revealed the following additional information about these isolates:

1) *Vibrio fetus* cultures which comprised groups I and II (O-3, H-38, O-5, and O-15) each possessed a single antigen in their saline and phenol fractions, respectively.

2) The antigens from *V. fetus* isolates in groups I and II were unrelated, as an independent reaction was demonstrated.

3) The isolates in group III subjected to saline and phenol extractions (*V. fetus* O-21, B-8, H-33, and H-40) possessed multiple antigens:

a) Two antigens were present in the saline fractions of O-21 and B-8, 1 of which was identical to the single phenol fraction.

b) Two antigens were present in the phenol fraction of H-33, 1 of which was identical to the single saline antigen.

c) *Vibrio fetus* H-40 contained 2 antigens which appeared in both the saline and phenol fractions.

d) Cross-reactions existed between the following antigens: O-21S and O-3P; H-40S and O-15P; B-8S and H-38P; H-38P and H-33P; and H-40P and O-15P.

This study demonstrated the antigenic heterogeneity of *V. fetus* and the feasibility of using saline and phenol extracts to identify unknown isolates.

With the few isolates of *V. fetus* of bovine, ovine, and human origin studied, no antigenic pattern was found associated with source of isolation.

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SUMMARIO IN INTERLINGUA

Un Studio in Vitro de Extrahite Antigenos de Isolatos de *Vibrio fetus*

Con le utilisation del technica precipitinic de Oakley-Fulthorpe, extractos salin e phenolic de antigenos de isolatos de *Vibrio fetus* esseva studiate in lor reactiones con antiseros de conilio. Le studio demonstrava le heterogeneitate antigenic de *V. fetus* e le practicabilitate de usar extractos salin e phenolic in le identification de non-cognoscite isolatos.

Viste le pauco numerose isolatos de *V. fetus* de origine bovin, ovin, e human que esseva includite in le studio, nulle configuration antigenic esseva trovate associate con le fonte del isolation.

Scientific Notes

Inoculation of Microbes into the Intestinal Tracts of Small Animals

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THE ACIDITY of the stomach juice acts as a barrier to orally administered drugs which are labile at a low pH. In order to administer drugs orally, various enteric coatings have been formulated to delay the release of a drug until it reaches the small intestine where the pH is higher.[1] Stomach acidity also hinders manipulation of the gastrointestinal micropopulation, because only acid-resistant microbes and microbial spores reach the intestine by the oral route. Attempts have been made to alter the gastrointestinal micropopulation by feeding diets high in milk and milk products which contain acid-resistant lactobacilli spores [2] and by administering microbes in acid-resistant capsules which are labile to intestinal juices.[3]

This report describes a method of enteric microbial inoculation in a small experimental animal, the rat.

Materials and Methods

Dehydrated microbes from rumen fluid were used as inoculum. The fluid, obtained from a freshly killed 2-year-old steer, was filtered through 4 layers of cheesecloth and lyophilized. A thick slurry of the lyophilized microbes was prepared with a 1:1 mixture of lauric and palmitic acids at 60 to 70 C. The slurry was aspirated into a glass tube (4-mm. outer diameter) and chilled at 0 C. until hard. When it retracted from the inner surface of the tube, it was extruded and cut to inoculum size (4–5 mm.).

The effect of simulated gastric fluid [3] and simulated intestinal fluid [4] upon the inoculum was studied to determine the stability of the inoculum in stomach juice and the lability in intestinal juice. Disintegration of inoculum in the simulated fluids was studied at 37 C.; aerobic and anaerobic bacterial colony counts of the fluids were made to determine the viability of the microbes in the inoculum.

A piston-type applicator was used to place the inoculum into the esophagus (fig. 1). The applicator barrel was a glass tube (6 mm. outer diameter, 110 mm. long) and the piston was a 3- by 140-mm. glass rod which slid easily into the barrel. The delivery surfaces of the barrel and piston were ground smooth to preclude injury of the animal's mouth. A gum rubber sleeve around the distal end of the barrel gripped the piston lightly, preventing slippage during the manipulation which preceded inoculation. An enlargement near the end of the piston shaft controlled the extent of piston movement so that no more than 2 mm. of the piston protruded beyond the barrel when inoculation was accomplished.

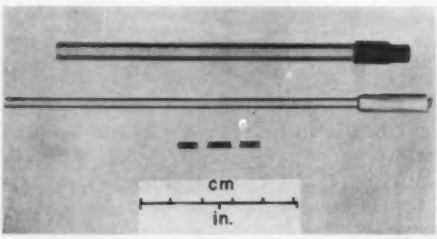

Fig. 1—Photograph of enteric inoculum applicator and inoculums.

The piston of the applicator was retracted to accommodate the inoculum which was held in the applicator barrel with a bit of margarine. Hand restraint was used to keep the rat in a natural position during inoculation. The applicator was worked gently to the rear of the mouth and the inoculum was placed in the esophagus by depressing the piston. Deglutination moved the inoculum into the stomach, where it became subject to gastric contractions and subsequent peristalsis. Additional inoculations caused little resistance after the initial introduction of the applicator.

Results and Discussion

In studies with simulated gastric and intestinal fluids, the inoculum remained intact in the gastric fluid, while disintegration in the intestinal fluid was noticed after 1 hour and was complete after

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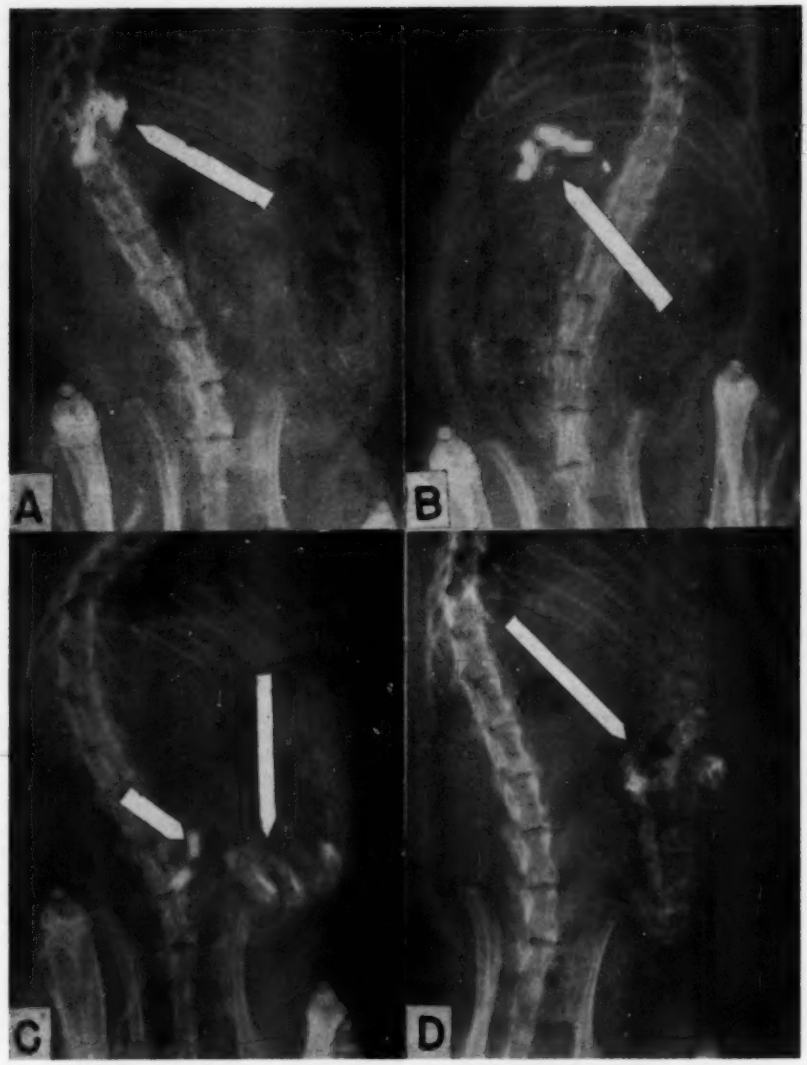

Fig. 2—Disintegration of enteric inoculum in the rat gastrointestinal tract. X-ray exposures made at 0, 3, 5 and 7 hours (A, B, C, D, respectively) after inoculation.

10 hours. After 24 hours of incubation at 37 C., inoculum in the gastric fluid was intact and unchanged; a putrid odor was detected in the intestinal fluid which had disintegrated the inoculum. No bacterial colonies developed from the gastric fluid, indicating that the microbes were held in the lyophilized state by the fatty acid vehicle. The colony count obtained from the intestinal fluid was of the order of 10^6, indicating that the microbes were released from the inoculum and resumed their growth.

Roentgenographs of 5 placebo inoculums, prepared with barium sulfate and the fatty acid mixture, were used to study the disintegration of inoculums in the rat gastrointestinal tract (fig. 2). The inoculums remained in the stomach for 3 to

4 hours without disintegrating. Retention time in the stomach varied from 2 to 7 hours, depending upon inoculum size, dietary satiation, and environmental adaptation. Enumeration of the inoculums was often difficult because of shadows cast by the inoculum nearest the x-ray film. The inoculums passed into the intestine after 4 hours, and disintegration at this site was indicated by loss of the inoculum shape and delineation of the small intestine. Disintegration of the inoculums in the gastrointestinal tract was complete after 7 hours, and the rat bowel was clearly outlined by the barium sulfate in the inoculums.

It is presumed that anaerobic microbial growth resumed following disintegration of the inoculums, since conditions in the intestine are ionically and nutritionally amenable to the existence of microbes.

Summary

1) A procedure was devised for using the oral route for microbe inoculations into the intestine of the small experimental animal.

2) The action of simulated gastric and intestinal fluids upon the microbe inoculum was studied. No action was observed with simulated gastric fluid, while complete disintegration of the inoculums in the simulated intestinal fluid occurred in 10 hours.

3) Roentgenography of placebo inoculums also indicated resistance to stomach juices and disintegration by intestinal juices.

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SUMMARIO IN INTERLINGUA

Inoculation de Microbios in le Vias Intestinal de Micre Animales

1. Esseva elaborate un methodo pro le uso del via oral in inoculationes de microbios in le intestinos de micre animales experimental.

2. Le action de simulate liquidos gastric e intestinal super le inoculo microbial esseva studiate. Nulle effecto esseva notate in le case del simulate liquido gastric, sed un complete disintegration del inoculos occurreva intra 10 horas in le simulate liquido intestinal.

3. Roentgenographia de inoculos placebo etiam monstrava resistentia contra le succos gastric e disintegration in le succos intestinal.

A Flocculation Test for the Detection of Antibodies Resulting from the Injection of an Extract of Hypoderma Larvae

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Manhattan, Kansas

EXPERIMENTS using ronnel (0,0-dimethyl 0 [2,4,5-tri-chlorophenyl] phosphorothioate) and Dowco 109 (0-[4-tert. butyl-2-chlorophenyl]-methyl methyl phosphoramidothioate) for the control of cattle grubs, Hypoderma spp., were made by Knapp et al.[4] over a 2-year period. The experiments indicated that absorption of the dead grubs by the host may cause development of antibodies against grub tissue, and that animals treated for grubs with systemic insecticides have fewer grubs the second season than do control animals.

Peter and Gachtgens [5] reported that cattle more than 5 years old were observed to be free from grubs. These workers also developed a flocculation antigen from the alcohol-soluble portion of grubs.

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Contribution No. 175, Department of Veterinary Medicine (Nelson) and Contribution No. 744, Department of Entomology (Knapp), Kansas Agricultural Experiment Station, Manhattan.

Based in part on a portion of a dissertation to be submitted by the senior author to the Graduate School, Kansas State University, in partial fulfillment of the requirements for the Ph.D. degree.

Sergent and Sergent [7] induced an active immune response in cattle by inoculating them with fluids expressed from grubs.

Kline [8] described a microscopic slide precipitation test for syphilis. The antigen used was made by coating cholesterol with the acetone-insoluble fraction of alcoholic heart extract.

Sussenguth and Kline [9] reported a useful flocculation test for trichinosis. The antigen was produced by using an alkaline aqueous extract of powdered Trichinella larvae coated on cholesterol crystals.

Materials and Methods

Second and third instar cattle grub larvae (Hypoderma spp.) were collected from the backs of infected cattle. The grubs were washed in distilled water and stored in a freezing chamber until used. An extract was prepared by grinding the grubs in a mortar; physiologic NaCl solution was added at the rate of 4.0 ml. per grub and sterile sand was used to facilitate grinding.

Two rabbits were given 3.5 ml. each of the grub extract intraperitoneally. A 350-lb. heifer was given two 20.0-ml. intraperitoneal inoculations 9 days apart; 20 days later, a third dose of 100.0 ml. was given. The heifer was exsanguinated 15 days after the third dose. Blood samples were taken from both rabbits 14 days after injection. The blood was allowed to clot, the serum was then removed from each sample and pooled. A 5 per cent aqueous phenol solution was added to the serum at the rate of 0.05 ml. per milliliter of serum. The serum was then placed in a water bath at 56 C. for 30 minutes and stored in a refrigerator until it was used. Immediately before use, the serum was reheated to 56 C. for 5 minutes.

Second and third instar larvae were ground in a mortar. Absolute ethyl alcohol was added at the rate of 1 ml. per larva. This suspension was held at 37 C. for 14 days. The supernatant fluid was then removed as soluble antigen and stored at 37 C. until used.

Other methods of making the soluble antigen from grubs were tried; i.e., by use of a ball mill, a microhomogenizer, and ultra sound on a homogenized mixture. None gave comparable results.

The quantities of reagents used were 0.1 ml. of lecithin, extracted from beef muscle, as a 1 per cent solution in absolute alcohol; 1.0 ml. of cholesterol (C.P.), ash-free and precipitated from alcohol, as a 1 per cent solution in absolute alcohol; 0.85 ml. of distilled water, deionized and then glass-distilled and deionized again, pH 7.1; and 2.5 ml. of 1.0 per cent and 0.85 per cent solutions of salt prepared by adding NaCl (C.P.) to distilled water prepared according to Eagle.[1]

A microscopic tube-flocculation test was used for antibody detection. The method of preparation of the antigen emulsion was the same as that described by Hunter and Colbert,[2] using 1 ml. soluble antigen.

The serum dilutions made in physiologic NaCl solution were 1:1, 1:2, 1:4, 1:6, 1:8, 1:10, and 1:20. Serums used as negative controls were obtained from a young rabbit and a calf. The control serum was handled like the positive serum. After 0.5-ml. quantities of diluted serum and undiluted serum were placed in separate test tubes, 0.5 ml. of the antigen emulsion, diluted 1:4 in 1 per cent NaCl solution, was added to each test tube. The tubes were shaken 10 minutes on a Kahn shaker and then centrifuged for 10 minutes at 2,000 r.p.m. (International centrifuge, size 1, type SB), after which they were again shaken 1 minute.

The tests were read by holding the tubes in front of a reading lamp with a black background. To facilitate reading, the tubes were given a swirling motion. A positive test was indicated by flocculation of the cholesterol crystals; whereas, no flocculation indicated a negative test. To prove the specificity of the test, 1 ml. of the alcoholic antigen emulsion was dried free of alcohol by boiling under vacuum. The residue was resuspended in 1 ml. of distilled water. One milliliter of the positive rabbit serum was added to the antigen solution; centrifugation removed the precipitate. This mixture was held at 37 C. for 1 hour and then shaken on a Kahn shaker for 30 minutes. A 1:1 dilution of the positive rabbit serum was made in distilled water. This dilution was the same as the antibody-adsorbed serum. The flocculation test was performed on the antibody-adsorbed serum and on the nonadsorbed serum.

Results

The serums from the rabbits and the heifer were both titrated to a dilution of positive at 1:8. Subsequently, the serum pooled from the rabbits that had been given injections of grub material produced a titer positive at 1:20. The negative bovine serum remained negative. The negative rabbit serum remained completely negative at titers of over 1:6. There was some clouding that could not be called positive or negative in the lower dilutions. This appeared to be limited to specific antigens. The titer in serum from the heifer was demonstrated only when the serum was less than 72 hours old. The highest recorded titer for the heifer was on the day she was exsanguinated. The rabbit serum retained its titer, although the flakes of the cholesterol crystals were smaller in the older serum.

Results of the flocculation test performed on the antibody-adsorbed positive serum and the nonadsorbed positive serum were negative and positive, respectively.

It was found that the soluble antigen could not be stored in a refrigerator because the antigen portion apparently precipitated out. If warmed to 37 C., the antigen was found to be satisfactory.

Discussion and Summary

The work completed thus far indicates the presence of antibodies in the serum of both heifers and rabbits inoculated with a grub (Hypoderma spp.) extract. It has been shown that the antigen will adsorb the antibody from the antibody-containing serum, thus proving the specificity of the antigen. It was also found that bovine serum stored more than 72 hours had a drop in titer. A drop in serum titer was also recorded by Roznowski,[6] who developed a flocculation test for the antibody of Salmonella pullorum.

Work is being initiated to increase the sensitivity of the test, because at the present time the test has produced no results in bovine serum collected in the field.

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SUMMARIO IN INTERLINGUA

Test de Flocculation pro le Detection de Anticorpore Resultante del Injection de un Extracto ab Larvas de Hypoderma

Le hic reportate investigationes, in tanto que illos ha essite completate, indica le presentia de anticorpore in le sero de conilio e vacca virgine post inoculation de extractos de larva de Hypoderma spp. Ha essite monstrate que le antigeno adsorbe le anticorpore ab le sero in que iste anticorpore es continite, de maniera que le specificitate del antigeno es provate. Esseva etiam trovate que sero bovin que es preservate durante plus que 72 horas possede un reducite titro. Un reduction del titro seral esseva etiam notate per Roznowski,[6] qui disveloppava un test de flocculation pro le anticorpore de Salmonella pullorum.

Ha essite initiate effortios a augmentar le sensibilitate del test. Usque nunc illo ha producite nulle resultatos in sero bovin colligite in le campo.

Meetings . . .

• • • Fourth International Congress on Animal Reproduction. The Hague, June 5-9, 1961. Secretariat, Burgemeester de Monchyplein 14, The Hague, Netherlands.

• • • Ninth International Conference on Spectroscopy. Lyons, June 5-10, 1961. Groupement pour l'Avancement des Methodes Spectrographiques, 1 rue Gaston-Boissier, Paris 15e, France.

• • • FAO Far East Meeting on Animal Production and Health. Bangkok, June 5-15, 1961. International Agency Liaison Branch, Office of the Director General, Viale delle Terme di Caracalla, Rome, Italy.

• • • Eighth International Congress of Animal Production. Hamburg, June 12-16, 1961. Koblenzerstr. 176, Bonn, Germany.

• • • FAO Subcommittee on Animal Health for Near East. Ankara, June 14-16, 1961. International Agency Liaison Branch, Office of the Director General, Viale delle Terme di Caracalla, Rome, Italy.

• • • FAO/International Office of Epizootics, Meeting on Emerging Diseases. Ankara, June 19-24, 1961. International Agency Liaison Branch, Office of the Director General, Viale delle Terme di Caracalla, Rome, Italy.

• • • Sixth Congress, International Association on Quaternary Research, Warsaw, Summer, 1961. Congress Secretariat, Nicholas Copernicus University of Torun, Torun, Poland.

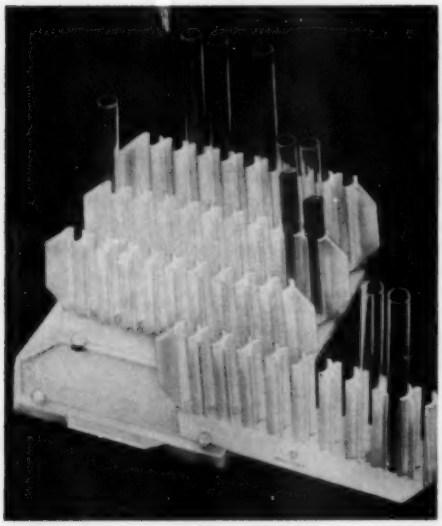
• • • FAO Meeting on Radioisotopes in Animal Nutrition and Physiology. Copenhagen, June, 1961. International Agency Liaison Branch, Office of the Director General, Viale delle Terme di Caracalla, Rome, Italy.

Research Tools

Further information on these products may be obtained by writing to: Research Tools, American Veterinary Medical Association, 600 S. Michigan Ave., Chicago 5, Ill.

Plastic Test Tube Rack

An Italian-designed plastic finger holding principle permits full view of test tube contents in a new test tube rack produced by the Nalge Co., Inc.

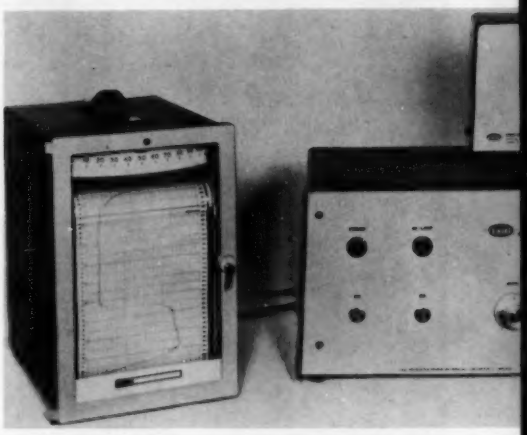


The rack, as well as the bases, are made of virgin, high-density polyethylene that is unbreakable and chemically inert.

Immersible in water baths, the test tube rack is easy to clean, light in weight, but stable and compact enough to fit into shaking machines. The supports are available as free-standing units, with capacity for nine 18-mm. or ten 16-mm. test tubes, or with plastic bases designed to hold two- or four-rack units.

Ultraviolet Analyzer System

A new system for the automatic analysis of flowing effluent from chromatography and electrophoresis columns has been designed by LKB Instruments, Inc. This analysis is possible through the interconnection of three instruments—an ultraviolet absorptiometer, a recorder, and a fraction collector.



The absorption of effluent is detected by the absorptiometer and the curve recorded on the recorder. A special "event marker" built into the recorder allows each test tube of fraction collector to be automatically indicated on the chart. Correlation between test tubes containing relevant sample and the indicating chart recording is practically immediate. Spectrophotometric analysis is then possible by reading a few test tubes.

Vacuum Refrigerated Centrifuge

A vacuum-type, refrigerated centrifuge capable of spinning up to 1,500 ml. (6 x 250 ml.) at 60,000 x g and 3,300 ml. (6 x 550) at 33,000 x g is now being produced by Lourdes Instrument Corp. This centrifuge also interchangeably accommodates and spins smaller rotors with 400 ml. (8 x 50 ml.) and 360 ml. (24 x 15 ml.) at forces up to 51,000 x g.

The model features automatic rotor acceleration for adjustable speed. Electrodynamic braking provides fast rotor deceleration without strain on moving parts, and an automatic time-relay permits the rotor to stop smoothly without disturbing the sediment.

With the refrigeration design, the rotor can be operated with temperatures as low as −10 C. during extended high-speed runs, or at −20 C. during shorter runs or at lesser speeds. Rotors may be quickly precooled. When operating under a vacuum, material temperatures can be maintained within 1 C. for temperatures as low as −10 C.

The centrifuge has a slanted control panel, continuous-reading electric tachometer, two-hour synchronous timer, temperature setting and indicating device, and automatic acceleration controls and pilot lights.

Miniature Resistance Thermometer

Minco Products, Inc. announces a new miniature resistance thermometer. Measuring only 0.138 in. in diameter and 0.4 in. in length, this hermetically-sealed temperature sensor has an accuracy of better than 0.05 degree F. The accuracy, small size, and rugged design of this sensor suit it to medical uses where highly precise temperature measurement, monitoring, or control is required.

Whereas most resistance thermometers have wire-wound elements supported on a mandrel or card with the resistance wire taut, this platinum sensing element is fully annealed for stability and suspended inside the hermetically-sealed metal case. This eliminates inaccuracies due to the physical strain set up in the resistance wire.

Plastic Laboratory Bottles

A new line of plastic bottles and closures, able to withstand repeated laboratory use, has been developed by Nalge Co., Inc. These bottles have strong, uniform walls, stable bases, and strong threads. The closures have semi-buttress threads, prominent outside ridges for easy manipulation, and inside sealing rings.

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